



## Molecular medicine techniques in the detection of viral hepatitis

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### ABSTRACT

Viral hepatitis is a major concern in the public health scenario which has been the root cause of serious adverse events affecting the human population. Molecular diagnostics has paved the way for an incredible role in the advancement of the prognosis and the diagnosis of viral hepatitis. Significant improvements and modifications in the techniques have led to a breakthrough in identifying the various reasons underlying the severity of the various hepatitis viruses causing acute and chronic infections besides cancer. The molecular biological analysis of the infectious agents involves detailed investigation of their genomes and their genomic products. Polymorphism studies have unleashed many important associations of the diseases with the various genetic variants. Besides these, recombinant DNA technology, modified sequencing technologies, rational drug designing have revolutionized the clinical sphere with their varied applications along with an immense impact on human health. Through the development of novel genetic methodologies, including a better perception of pathogen biology, pathogenic mechanisms, recent advances in vaccine development, scheming new therapeutic drugs and optimization of diagnostic tools, viral hepatitis caused by viral infections are now better controlled. In this brief review we discuss the developments in the various diagnostic tools in the detection of the viral infections inflicting the liver in the preceding years along with some recent upcoming techniques which may revolutionize the treatment and diagnosis of viral hepatitis caused by various viral infections.

**Keywords:** Viral Hepatitis, Molecular Tools

**Core Tip:** The review addresses some interesting aspects of molecular biology in the detection, isolation and management of viral hepatitis. With a distribution of varied genotypes and genetic variants, these molecular techniques have been an eye-opener in identifying people who are at higher risk of developing severe chronic liver disease, and can unknowingly transmit the infection to other people thus becoming a primary part of basic as well as clinical hepatology. Genetic tests can be helpful in substituting invasive procedures. In the next millennium, molecular biological methods will become increasingly imperative in clinical and laboratory medicine imposing a deep impact on human health.

### INTRODUCTION

Viral hepatitis is an important cause of public health burden in the present day world. Molecular methods allow the premature and/or specific detection of hereditary infectious and malignant liver diseases. In

addition, such analyses have increasingly lead to a better perception of the pathogenesis of the various liver diseases which in turn had a burden on patient supervision, including the presymptomatic identification of patients at risk, the correct staging

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of the disease, and the follow-up of patients undergoing therapy. Thus, molecular biology is increasingly becoming a fundamental part of basic as well as clinical hepatology. Methods such as the polymerase chain reaction are changing the way physicians analyze and scrutinize patients with viral hepatitis. The detection system should have the characteristics of a Detection System. A good detection system should have 3 qualities: (a) Sensitivity (b) Specificity (c) Simplicity

Sensitivity means that the test must be able to detect very small amounts of target even in the presence of other molecules. Specificity requires the test to yield a positive result specific for the target molecule only. Simplicity implies the test to be able to run efficiently and reasonably on a routine basis.

The molecular biological investigation of infectious agents involves scrutinizing their genomes and their genomic products. Molecular biology methods may therefore allow us to study either DNAs or RNAs. The study of genomic RNAs (viruses) or messenger RNAs (all infectious agents) is used increasingly in infectious disease pathology.<sup>1</sup> The discovery by Baltimore<sup>2</sup> and Temin and Mizutani<sup>3</sup> of retroviral reverse transcriptase, which directs the synthesis of cDNA from a RNA template, was of fundamental significance in modern molecular biology. PCR, conceived of by Mullis<sup>4,5</sup> has also revolutionized modern molecular biology by allowing the in vitro enzymatic amplification of large amounts of DNA from a small number of molecules. The use of thermostable DNA polymerases, such as Taq from *Thermus aquaticus*, has made PCR a rapid, reproducible, and semi-automated procedure.<sup>5</sup> The area of viral hepatitis diagnosis has not escaped the impact of the discoveries of reverse transcriptase and PCR. Antisense oligonucleotides<sup>6</sup> can be used to inhibit the expression of genes essential for the replication of hepatitis viruses. Ribozymes are catalytic RNA molecules that can be used for similar purposes as antisense oligonucleotides.<sup>7</sup>

## HEPATITIS

"Hepatitis" means inflammation of the liver. Viral hepatitis is most often caused by one of several viruses, such as –Hepatitis A virus (HAV),

Hepatitis B virus (HBV), Hepatitis C virus (HCV), Hepatitis D virus (HDV), Hepatitis E virus (HEV); and Hepatitis G virus (HGV). Viral hepatitis carries a heavy burden on the health care system because of the costs of treatment of liver failure and chronic liver disease. Approximately 1, 000, 000 people die each year (~2.7% of all deaths) from causes related to viral hepatitis; most commonly liver disease; including liver cancer.<sup>8</sup> In many countries, viral hepatitis is the leading cause of liver transplants. Millions of people are living with viral hepatitis, and millions more are at risk. Most people who were infected long ago with HBV or HCV are unaware of their chronic infection. They are at high risk of developing severe chronic liver disease and can unknowingly transmit the infection to other people.

## Hepatitis A

It has a worldwide distribution with endemic and epidemic forms. It occurs mainly in children and young adults. In a large majority of patients it is asymptomatic. It is an enterovirus which is non-enveloped and single stranded RNA virus. Although HAV is primarily shed in feces, there is a strong viremic phase during infection which has allowed easy access to virus isolates, and the use of molecular markers to determine their genetic relatedness.<sup>9</sup>

### Diagnosis:

- 1) Serological Diagnosis of HAV:
  - ELISA for anti-HAV IgM is done according to manufacturer's instructions which can detect the acute antibody response with a rise in IgM and simultaneous rise in IgG.
- 2) Nucleic Acid Detection: The viral nucleic acid is detected by:
  - a) HAV RNA- RTPCR: By reverse transcriptase PCR, viral nucleic acid is detected by use of specific primers. Primers specific for HAV RNA are used, one of which is described by:
  - b) HAV viral Load- Real time PCR: HAV viral load copies are detected by real time PCR using Real time PCR Kit as described by manufacturer's instructions (Genome Diagnostics, Sydney).



Newly designed real-time TaqMan SH-Poly and SH-Prot primer and probe systems have been developed for the detection of HAV RNA in clinical, environmental, and food samples which may lead to rational decisions in public health policies.<sup>10</sup>

### Hepatitis B

Polymerase chain reaction (PCR) amplification<sub>4</sub> of HBV DNA is also relatively easy as HBV is a double stranded DNA virus whose genome is fairly stable in blood and tissue. Sequencing of amplified DNA can be performed to identify mutant viruses of clinical significance. Truncating mutations in the HBV precore gene have been identified that prevent secretion of the e antigen but allow the continued assembly of infectious virus.<sup>11</sup> Even though the serological assays become negative for HBeAg, the mutant strains may be replicating which may add to the woes of the patient.

HBV is the major cause of acute and chronic liver diseases, and persistent HBV infection is associated closely with the development of cirrhosis and hepatocellular carcinoma (HCC), accounting for 1 million deaths annually.<sup>12</sup> The clinical outcome of HBV infection varies greatly from acute self-limiting disease and inactive carrier state up to chronic liver disease including liver cirrhosis and hepatocellular carcinoma (HCC).<sup>13</sup> The reasons why some patients with chronic hepatitis B infection progress to HCC are unknown.

However, the advent of molecular biology-based techniques has added a new dimension to the diagnosis and treatment of patients with chronic HBV infection. HBV is a partially double-stranded DNA virus. Therefore, viral genomic sequences can be amplified directly by PCR without reverse transcription. Regarding serological diagnosis, hepatitis B differs from hepatitis C in that viral protein antigens of HBV can be readily detected in serum. Hepatitis B surface antigen is detectable in virtually all infected individuals, and the hepatitis B e antigen is detected in most patients who have high amounts of viral replication. Accordingly, nucleic acid tests are not necessary to diagnose viral infection with HBV in the clinical laboratory. In some instances, however,

amplification of viral nucleic acid sequences from serum is helpful in assessing HBV infected individuals.

### Diagnosis:

Because viral load may be predictive of response to treatment and prognosis, it may sometimes be useful to use semi quantitative PCR to estimate serum viral concentrations. Assays utilizing bDNA can also be used to quantify HBV DNA in serum. Sequencing the HBV DNA amplified by PCR can be useful for identifying mutant viruses of clinical significance. For example, mutations in the HBV precore region of the genome have been identified that prevent transcription of the e antigen but allow the continued assembly of infectious virus. In a previous study the presence of different types of HBV mutations, age, sex, HBeAg status, and viral load was found to increase significantly the risk of HCC development in India.<sup>14</sup> The occurrence of multiple mutations may represent an HBV immune surveillance escape strategy that contributes to the multi-step hepatocarcinogenesis process.

### Hepatitis C

Hepatitis C virus is the major etiological factor among the NANBH. Hepatitis C virus is a sole member of the genus hepacivirus in the family Flaviviride. The hepatitis C virus (HCV) nonstructural 3 (NS3) protein is composed of an amino terminal protease and a carboxyl terminal RNA helicase of which NS3 contains major antigenic epitopes.<sup>15</sup> More than 210 million people are affected per year by hepatitis C virus (HCV) worldwide and has been recognized as a major cause of chronic hepatitis, end-stage cirrhosis and hepatocellular carcinoma.<sup>16</sup> Hepatitis C virus infection is being recognized as a major health problem in 170 million people infected worldwide. Approximately, 50-70% of HCV infected patients develop chronic liver disease. The hepatitis C virus (HCV) was identified in 1989 by investigators at Chiron Corporation.<sup>17</sup> HCV-RNA levels are detectable after 1-2 weeks of exposure, and before elevation of transaminases and development of clinical signs. In approximately 75%–85% of cases, HCV endures as a chronic infection, introducing infected persons at risk for liver cirrhosis, an extrahepatic complication, and hepatocellular carcinoma (HCC) that develop gradually following onset of infection.<sup>18</sup> The rate of



evolution to chronicity after acute exposure to HCV was 92% in patients exposed to HCV genotype 1b infection, compared with 33% to 50% in patients exposed to other genotypes which may perhaps elucidate the role of viral factors, including the HCV genotype, in the development of chronic infection following acute exposure to HCV.<sup>19</sup> Only patients with detectable HCV RNA should be considered for pegylated interferon alfa and ribavirin therapy, and the HCV 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.<sup>20</sup>

#### **Diagnosis of HCV infection:**

The diagnosis of HCV infection can be done by the following:

Nucleic Acid tests are as follows:

- a) 3<sup>RD</sup> Generation ELISA (anti HCV antibody)
- b) Recombinant Immunoblot assay (RIBA 3 test).
- c) RTPCR for demonstration of HCV RNA
- d) Real time PCR for determination of viral load.

Values for viral load can be used to assess a patient's response to treatment.

By using different PCR primers and probes to amplify and detect different sequences from various isolates, routine HCV genotype analysis is possible in the clinical laboratory.<sup>21</sup> The HCV genotype, HCV RNA, HCV core antigen, and anti-HCV antibodies are the four biologic markers currently used in hepatitis C, besides, acute and chronic hepatitis C are diagnosed by anti-HCV antibody (enzyme immunoassay), and HCV RNA detection with sensitive molecular biology techniques.<sup>22</sup> Since the HCV genome is RNA, reverse transcription is performed before HCV complementary DNA sequences can be amplified by PCR after the products can be observed by agarose gel electrophoresis. Care must be taken in the clinical laboratory to avoid contamination when using reverse transcription-PCR to detect HCV RNA; however, the standardization of this method in excellent clinical laboratories has made it the standard for the detection of HCV infection RT-PCR; can also be used to determine the HCV genotype. At least 6 major genotypes and 11 subtypes of HCV have been identified on the basis of genomic sequence

differences. Several methods exist for determining HCV genotype, including RT-PCR followed by direct sequencing, RTPCR with genotype-specific primers, restriction fragment length polymorphism (RFLP) analysis of PCR-amplified DNA sequences. Some of these genotyping assays are now performed in clinical laboratories; combined with estimates of serum viral load; knowledge of the infecting genotype is useful in predicting prognosis and response to therapy. Besides these, second and third generation line probe assays like INNO-LiPA HCV II method using INNO-LiPA HCV II kit (Belgium Innogenetics) as described earlier are being used in the genotyping of HCV infection.<sup>23</sup> Sequence variations are more subtle than those between different genotypes, e.g., point mutations that cause amino acid substitution in the NS5A gene product, also correlate with response to interferon therapy.

In a previous study involving TLR 3 promoter region single nucleotide polymorphism and HCV infection utilizing semi quantitative PCR and insilico analysis of promoter region by us<sup>24</sup> presence of two previously unreported SNPs at -705 and -288 promoter region of TLR3 was reported and SNP of -705 was associated with chronic HCV infection. Hence, it can be observed that not only the clinical diagnosis but the molecular tools can be used in assessing the genetic variations in the patients with HCV infection.

#### **Hepatitis E**

Hepatitis E virus (HEV) is an important cause of epidemic and sporadic acute viral hepatitis in many developing countries, including India.<sup>25</sup> Hepatitis E, a positive-sense single-stranded RNA virus, approximately 7.2 kb in length had been currently placed in the genus Hepevirus and is the only member of the family Hepeviridae.<sup>26</sup> Using genomic sequence analysis, HEV isolates from human and other mammals have been divided into four genotypes, namely 1, 2, 3 and 4, and at least 24 sub-genotypes (1a-1e, 2a-2b, 3a-3j and 4a-4g).<sup>27</sup> Pregnant women with jaundice and acute viral hepatitis caused by HEV infection have worse fetal and obstetric outcomes, and higher maternal mortality compared to other types of viral hepatitis.<sup>28</sup> Studies from various developing countries have shown that the incidence of HEV infection in pregnancy is high, and a



significant proportion of pregnant women can progress to fulminant hepatitis with a mortality rate varying from 30–100%.<sup>29,30</sup> The reason behind the high mortality in the pregnant patients has been attributed to folate deficiency<sup>31</sup>; NK kappa B signaling regulating at the transcriptional level through p50 subunits<sup>32</sup>, viral load of HEV during the infection<sup>33</sup>, reduced expressions of progesterone and progesterone-induced-blocking factor and the high viral load of HEV;<sup>34</sup> but, still the scenario remains unclear. So with the use of modified real time PCR assays, molecular cloning techniques, and cell culture facilities now available, novel mechanisms of the virus can be unleashed.

#### Diagnosis:

Diagnosis of HEV can be done by ELISA for anti-HEV IgM according to manufacturer's instructions. Anti HEV IgG antibody ELISA can also be done to detect prior exposure to Hepatitis E virus infection.

#### Nucleic acid assays:

HEV viremia can be detected by HEV RNA- RT PCR using appropriate primers,<sup>35</sup> and is the confirmatory test for HEV detection. HEV Viral load can be estimated by HEV Real time PCR using a viral load estimation kit which had led to a new finding of its correlation with the mortality in patients with acute liver failure during pregnancy.<sup>33</sup> Two real-time PCR methods had been developed; one, a TaqMan, and another Primer-Probe Energy Transfer (PriProET) assay provide new tools to study HEV biology, including virus-host interactions, and transmission between various host species besides detecting the virus in the food chain.<sup>36</sup>

Years of extensive research have provided us two safe and highly effective vaccines; but, they have not been circulated in the market yet due to the lack of large population based studies;<sup>37</sup> but, new recombinant technologies may further pave the development of a much effective safer vaccine.

**Table 1 Mode of Transmission in HAV, HBV, HEV and HCV**

Virus	Incubation period	Transmission mode	Vertical transmission
HAV	15-50 days its symptoms last for 2-3 weeks	Faecal Route (a) Person to person contact (b) Eating infected food (c) Drinking untreated water	No
HBV	Average 75 days, may vary from 30-180 days.	Unsafe sexual intercourse, transfusions of HBV-infected blood and blood products, contaminated injections during medical procedures, and sharing of needles and syringes among injecting drug users.	Mothers to infants at the time of birth, or from family members to infants in early childhood.
HCV	6-10 weeks	Blood products, unsafe sexual practices, more commonly in injection drug users, homosexuals and even through tattooing.	Less than 10 % in the pregnancies
HEV	3-8 weeks with a mean of 40 days.	Faecal-oral route, waterborne disease, zoonotically	Yes

#### Hepatitis Delta Virus

The hepatitis delta virus (HDV) consists of a small (~1700 nucleotides), negative strand, circular RNA molecule, folding into an unbranched, rod-like

structure due to about 70% self-complementarity.<sup>38</sup> The HDV RNA genome contains two complementary ribozyme motifs (i.e. delta Rz), and has a single open reading frame on antigenomic HDV RNA encoding two viral proteins (HDAg-S).



These two proteins are mostly identical in sequence except that the large HDAg (HDAg-L) contains 19 additional amino acids at its C-terminus resulting from RNA editing of the antigenomic HDV RNA located on the termination codon of the small HDAg (HDAg-S) gene to a tryptophan codon.<sup>39</sup> HDAg-S is essential for HDV replication<sup>40</sup>, while the HDAg-L was reported to be required for virion assembly and to be an inhibitor of HDV replication.<sup>41,42</sup> HDV requires the hepatitis B virus (HBV) envelope proteins for dissemination.<sup>43</sup> However, HDV replication is entirely independent of the helper virus and uses host-encoded RNA polymerase II (RNAP II) to replicate its RNA genome without DNA intermediates.<sup>44, 45</sup> it was previously reported that HDV seroprevalence in hepatitis B virus-related liver disease was higher in HCC cases (33%) compared to cirrhosis cases (15%) and chronic hepatitis cases (8%), respectively.<sup>46</sup> Worldwide 15 million people positive for hepatitis B are also infected with hepatitis D.<sup>47</sup> it also has oncogenic abnormalities.<sup>48, 49</sup>

#### **Diagnosis:**

HDV is diagnosed by the presence of anti-HDV antibody in the serum using specific ELISA assay.<sup>50</sup> Using a modified form of RNA ligation-mediated amplification of cDNA ends; it was shown that the 5' end was located at nt 1212.<sup>51</sup>

#### **Hepatitis G**

In independent work, investigators at Genelabs Technologies determined the complete genomic sequences of two isolates of a flavivirus that they called HGV which is now known as GB-C virus Type C.<sup>52</sup> HGV is essentially identical to the GB-C virus and is related to HCV, GB-B, and GB-A GBV-C is a member of the Flaviviridae family and is phylogenetically related to hepatitis C virus; but, replicates primarily in lymphocytes; and poorly, if at all, in hepatocytes.<sup>53</sup> In 1995 and 1996, a new human hepatotropic virus was identified.<sup>54,55</sup> To isolate HGV, the investigators at Genelabs screened a cDNA expression library constructed from the plasma of a patient with chronic hepatitis C. Immunoscreening of the expression library with the patient's serum identified several cDNA clones encoding HCV polypeptides as well as clones encoding other related but unique polypeptides. From the unique cDNA

clones, an anchored PCR method was used to amplify overlapping clones for the entire viral genome. Using RT-PCR, these workers identified HGV sequences in 13% of 38 US patients with non-A, non-B, non-C, non-D, non-E hepatitis and in 18% of patients with HCV infection. The mutation rate of the GBV-C genome has been estimated at  $10^2$  to  $10^3$  substitutions/site/year.<sup>56</sup>

The prevalence of HGV in the general population in India was found to be 4% but significantly a higher frequency (46.6%;  $p < 0.001$ ) of HGV was observed in commercial blood donors.<sup>57</sup> In another finding using reverse transcriptase polymerase chain reaction, HGV infection was commonly observed in both cirrhosis patients as well as healthy blood donors and a significant association of the virus with blood transfusion was observed which indicated of a parenteral route of transmission.<sup>58</sup> Recently, it has been reported that using digital PCR, a microfluidic device, viral load of GB Virus Type C RNA virus was estimated directly in a digital format without the need for a standard curve.<sup>59</sup>

Although it has been associated with acute and chronic hepatitis; ELISAs based on HGV/GB-C polypeptides are able to detect antibodies against this virus in the blood supply and in infected individuals. GBV-C infection has not been convincingly associated with any disease; however, several studies found an association between persistent GBV-C infection and improved survival in HIV-positive individuals and hence it is being termed as a "good boy virus".<sup>60</sup>

#### **Mutation Detection**

Knowledge of genes and mutations that cause various viral hepatitis will revolutionize the manner in which these conditions are diagnosed. Genetic tests can replace invasive procedures, such as liver biopsy, to rule out rarer conditions. It is relatively easy to detect a specific mutation that causes a disease. Detection of a mutation in a family member when the mutation is already known in a relative is also fairly simple. In these instances, RFLP analysis or specific oligonucleotide probes can be used to detect the mutation in total genomic DNA or DNAs amplified by PCR.





### Recombinant Drugs and Vaccines

Recombinant DNA technology has led to the production of drugs and products for the treatment and prevention of liver diseases. Notable among these products are interferons for the treatment of viral hepatitis and vaccines to prevent hepatitis B. Recombinant interferon  $\alpha$ -2b is effective in treating chronic hepatitis B and C.<sup>61</sup> Interferon  $\alpha$ -2b was the first recombinant drug approved by the US FDA for treatment of hepatitis and has been widely used. Human HBV vaccines have been produced by recombinant DNA technology. Vaccines composed of hepatitis B surface antigen particles expressed from recombinant DNA in budding yeast have repeatedly been demonstrated to be effective. Recombinant vaccines that protect against other hepatitis viruses may soon be developed. Preliminary results in cynomolgus monkeys suggest that vaccination with a recombinant protein representing part of the viral capsid antigen may be protective against hepatitis E to which no vaccine has been marketed.

### Rational Drug Design

The ability to express portions of proteins from recombinant cDNA clones provides the opportunity to determine their structures. Structure determination can lead to the rational development of drugs that can, for example, inhibit an enzyme or bind to a receptor. The first steps towards rational drug design have recently been reported for the NS<sub>3</sub> protease of HCV. This protease cleaves nonstructural polypeptides from the HCV polyprotein and is essential for viral replication. Workers at Vertex Pharmaceuticals and Agouron Pharmaceuticals have used x-ray crystallography to determine the structure of the NS<sub>3</sub> protease. Standard molecular biological methods were used to obtain sufficient quantities of protein for crystallization. Based on the three-dimensional structures determined in these studies, drugs can be rationally designed to inhibit this enzyme, which is essential for viral replication. Similar methods should be useful in developing other antiviral drugs and possibly even drugs that can stimulate defective enzymes in inherited diseases.

### Gene Therapy

Gene therapy holds promise as future treatment for liver diseases, including viral hepatitis. In brief, two

types of gene therapy strategies can be used to target the liver: ex vivo and in vivo. In ex vivo gene therapy, hepatocytes are removed from the patient and cultured in vitro. The desired expression vector is introduced into the cultured hepatocytes. In liver-directed in vivo gene therapy, genetic material is introduced into hepatocytes by gene transfer vectors that function after being introduced directly into the patient. Vectors for hepatic in vivo gene therapy include DNA complexed with proteins, DNA in liposomes, naked DNA and hepatotropic viral vectors such as those based on adenovirus.

### Other Strategies

Different strategies have been applied to a number of malignant and infectious diseases like use of particular ribozymes, siRNAs and antisense oligonucleotides have been experimentally explored to treat hepatitis B virus (HBV) and hepatitis C virus (HCV) infections and more recently HEV infections.

### Ribozymes

(‘ribonucleic acid enzymes’) were originally discovered as naturally occurring RNA molecules that catalyze the sequence-specific cleavage of RNA and RNA splicing reactions<sup>62</sup> which is the major attraction of the ribozyme concept since one ribozyme can cleave many target RNAs and its effect has been studied in cleaving HCV RNA.<sup>63,64</sup>

### RNA interference (RNAi)

It is a sequence-specific cellular antiviral defense mechanism, induced by double-stranded RNA, which had been used to investigate knockdown of several genes and the 3' cis-acting element (CAE) of HEV.<sup>65</sup> In HCV infection, inhibition of viral gene expression and replication has been shown in vitro in the replicon system.<sup>66</sup>

### Antisense nucleic acids

They are designed to specifically bind to RNA or mRNA, resulting in the formation of RNA-DNA (antisense oligodeoxynucleotides) or RNA-RNA hybrids (antisense oligoribonucleotides) with an arrest of RNA replication, reverse transcription or mRNA translation.<sup>67,68</sup> The antisense strategy has been successfully applied in vitro to HBV infection,<sup>69,70</sup> and to HCV infection.<sup>71,72</sup>



### Sequencing techniques

With the advent of Next generation sequencing (NGS) techniques and the decrease in the genome sequencing price, large number of sequencing data can be obtained about the various viral infections inflicting the liver and causing alarming damage. The first group to apply NGS to whole human genomes was Roche/454 in collaboration with Gibbs and colleagues at the BCM-HGSC, who reported the diploid genome of James D. Watson.<sup>73</sup>

Nowadays, full genomes are mapped and published nearly weekly, and with ever increasing pace and decreasing costs. NGS methods and platforms have developed during the last 10 years; and the quality of the sequences has reached a level where NGS is utilized in clinical diagnostics of humans.

The arrival of NGS technologies in the marketplace has changed the way we think about scientific approaches in basic, applied and clinical research. In some respects, the potential of NGS is akin to the early days of PCR, with one's imagination being the primary limitation to its use. The major advance offered by NGS is the ability to produce an enormous volume of data cheaply — in some cases in excess of one billion short reads per instrument run. Varied mutations occurring in the viruses affecting the disease severity are yet to come to light which might bring new insights into the diagnosis of the viral infections of the liver.

The next generation sequencing approach provides vital clues on the mutational background of genes concerned in signaling pathways in particular JAK/STAT, Wnt/ $\beta$ -catenin, p53 pathways and multiple chromatin regulator genes that drastically prop up hepatocarcinogenesis.<sup>74</sup> Next-generation sequencing is now primarily used for an array of applications in virology including virus discovery, exploration of quasispecies, viral progression, metagenomics, and analysis of antiviral resistance.<sup>75</sup> Next generation sequencing techniques have detected a considerable number of non-coding RNAs (ncRNAs) associated with HCC, mainly caused by HCV infection, have been found to be differentially expressed and to be implicated in pathogenesis of HCV-associated HCC.<sup>76</sup>

Besides these, informative data on the global gene expression pattern of HCV-related HCC and non-HCC counterpart compared to the normal liver tissues have been obtained by gene expression profiling assays using *high density microarrays*.<sup>77</sup> Compared with other molecular biology techniques applicable to anatomical pathology, In situ hybridization is better preferred by the scientists due to its similarity to immunohistochemistry and since it possess the unique advantage over other molecular biology techniques--largely based on probe hybridisation with nucleic acid extracted from homogenized tissue samples--of allowing localization and visualization of target nucleic acid sequences within morphologically identifiable cells or cellular structures.<sup>78</sup>

In recent studies, *Mass spectrometry (MS)* has emerged recently as a rapid, cost-effective, reproducible and accurate alternative approach.<sup>79</sup> MS-based molecular assays are highly acquiescent to mechanization and may provide a suitable platform for routine application to the inspection of HBV and HCV infections in the near future.

### CONCLUSION

In summary, molecular biology has already made a large impact on the diagnosis and treatment of liver diseases. In the next millennium, molecular biological methods will become increasingly important in clinical and laboratory medicine. While the recent developments in gene therapy for viral hepatitis are promising, various delivery, targeting and safety issues need to be addressed before these strategies will enter clinical practice. Creative research and hard work should lead to inexpensive diagnostic tests and effective treatments for many of the liver diseases that affect people throughout the world.

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