PROGRESS IN DIAGNOSIS OF VIRAL HEPATITIS A, B, C, D AND E

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Introduction

The information based on viral hepatitis has exploded during the past several years. In particular advances in molecular biology have widened our knowledge of hepatitis B and provided the first glimpses of an agent responsible for post-transfusion hepatitis.

Mainly five distinct viruses that cause viral hepatitis are currently recognized (hepatitis A, B, C, D and E viruses). Recent changes in the nomenclature contribute to the difficulty in keeping pace with developments concerning this group of viruses (Kurstak, 1993). Hepatitis A virus (HAV) and hepatitis B virus (HBV) have indeed retained their original names. The agent that causes post-transfusion non-A non-B hepatitis has been designated hepatitis C virus (HCV). The hepatitis delta virus, found only in patients with hepatitis B is known currently as hepatitis D virus (HDV). The agent causing epidemic non-A non-B hepatitis has been named hepatitis E virus (HEV).

All five viruses cause illnesses that cannot be distinguished on the basis of clinical findigs or results of physical examination, liver function tests or histologic study of liver biopsy specimens. By large serologic testing currently available for each type, except HEV, appears to be the mainstay and of great benefit in diagnosis. The current status, methods and future prospects of diagnosis of each type of viral hepatitis is discussed in this review.

Abbreviations: aa = amino acid; Ag = antigen; ALT = alanine aminotransferase; anti- = antibody (against); CEP = counterelectrophoresis; CP = complement fixation; EIA = enzyme-immunoassay; ELISA = enzyme-linked immunosorbent assay; HAV = hepatitis A virus; HBV = hepatitis B virus; HCV = hepatitis C virus; HDV = hepatitis D virus; HEV = hepatitis E virus; IAHA = immune adherence haemagglutination; IEM = immunoelectron microscopy; IF = immunofluorescence; IP = immunoperoxidase; ISTA = integrating sphere turbidimetric assay; PCR = polymerase chain reaction; RIA = radio-imunoassay; RPHA = reverse passive haemagglutination; RT = reverse transcription

Diagnosis of HAV infection

The cultivation *in vitro* of HAV and the subsequent development of immunoassays for the detection of anti-HAV IgM led to an easily performable approach to the rapid diagnosis of HAV infection from a single serum sample obtained any time during the acute illness or even during early convalescence. Molecular cloning of the HAV genome (Ticehurst *et al.*, 1983; Baroudy *et al.*, 1985) has yielded cDNA probes for use in the detection of HAV-RNA by cDNA-RNA hybridization. The currently available diagnostic tests for HAV infection are discussed below.

Rapid viral diagnosis

In vitro test in experimental animals to identify HAV antigen and antibody, and first- and second-generation *in vitro* tests (complement-fixation (CF), immune adherence haemagglutination (IAHA) contributed to rapid progress in HAV infection research during the last decade or so. However, third-generation radio-immunoassays (RIA) and enzyme-immunoassay (EIA) of high sensitivity and specificity have in fact effectively replaced them. These techniques could be utilized to detect HAV in clinical specimens and to monitor the presence of HAV during purification or *in vitro* cultivation to detect the presence of total antibody against HAV (anti-HAV) for seroepidemiologic studies and as an indicator of immunity, and towards the detection of anti-HAV IgM for rapid diagnosis of acute infection. These tests appear to be basically simple to perform and advantageous

in the sense that assays for anti-HAV are currently commercially available (Hollinger *et al.*, 1991).

Serological diagnosis

A diagnosis of acute HAV infection can be made by demonstration of HAV in tissues, body fluids or excretions or by proof of immune response to HAV. Since the viremic phase of HAV is rather short and the rarity in availability of samples before the onset of symptoms, serological testing for circulating HAV antigen and/or particles obviously is of limited value. The diagnosis of HAV by demonstration of a specific immune response seems to be main, suitable option. Classically, this is achieved by measurement of an increase in an appearance of serum anti-HAV between the acute and convalescence phases of illness in an interval of 4-6 weeks at least. Acute HAV is diagnosed by detecting anti-HAV IgM in serum taken during the acute illness (Bradley *et al.*, 1977). It reaches peak values within a few weeks of onset of symptoms, declining rapidly thereafter (Flehmig, 1979). Five months after onset of illness, 50% of patients appear to lose anti-HAV IgM (Hatzakis, 1984) while the majority have undetectable anti-HAV IgM within a year.

RIA and EIA techniques

Among the most practical methods known to detect anti-HAV IgM are modifications of the classical RIA and EIA techniques for detection or measurement of anti-HAV. A simplified procedure involves separation of the IgG and IgM fractions of sera by sucrose density gradient centrifugation (Swenson *et al.*, 1981) and subsequent determination of anti-HAV in the two fractions by EIA or RIA, but the cumbersome steps of centrifugation and fractionation of sera limits its clinical application. Another method used is the absorption of IgG from test serum by use of staphylococcal protein A with preferential binding for IgG (Bradley *et al.*, 1987). If the level of binding in RIA was not reduced by staphylococcal protein A absorption, the anti-HAV was considered to be primary of the IgM class. Applications of this approach have been limited by the suitability of only certain strains of *Staphylococcus* for IgG absorption, the fact that all IgG subclasses are not absorbed by staphylococcal protein A, poor reproducibility of the test and high frequency of false positives resulting from high levels of anti-HAV IgM in serum (Lofgren *et al.*, 1980).

Currently, an antibody class-capture approach forms the basis for the most popular and widely used assays for anti-HAV IgM. The anti-human IgM is used to coat the solid phase (microtiter well or plastic bead) followed by addition of test serum and subsequently the HAV and enzyme-conjugated for radioactively labelled ^{125}I-conjugated anti-HAV IgG. Hence, IgM molecules of all antigenic specificities may bind to the solid phase but the presence of anti-HAV IgM provides the link for the completion of the sandwich immunoassay (Duermeyer *et al.*, 1979). Provided that the anti-human IgM is heavy chain-specific, this approach is the most sensitive and specific (Mortimer *et al.*, 1981). Adopted by many investigators in either an RIA or EIA format this solid phase-bound antibody class-capture method is the one that is available commercially (Hansson *et al.*, 1981; Mortimer *et al.*, 1981). Anti-HAV IgM detectable with this type of assay appears within a few days of the onset of symptoms and reaches a peak within 1 to 3 weeks (Decker *et al.*, 1981). Generally anti-HAV IgM can be detected for at least 2-4 months after acute illness and in rare cases as long as for 6-12 months to allow optimal discrimination between very recent acute and past infection. Decker *et al.* (1981) included a 1:4000 dilution of the serum to be tested. Designed as such, the test currently commercially available (HAVAB-M and HAVAB-M EIA, Abbott Laboratories) is almost universally negative by 3 months after onset of acute illness. Although rheumatoid factor could interfere theoretically with this type of assay and yield false positive results in rare exceptions (Duermeyer *et al.*, 1979), this has not been encountered and essentially rheumatoid factor levels are rarely high enough to interfere at serum dilution of 1:4000. Recently, a fully automated microparticle enzyme immunoassay (MxHAVAB) has been developed for the detection of antibody against hepatitis A (Robbins *et al.*, 1991). MxHAVAB sensitivity reportedly is 18-25 U/I and is more sensitive than the commercial RIA or EIA, HAVAB and HAVAB EIA, repectively.

Current trends in molecular biotechniques

Molecular cloning of the HAV genome has been performed (Baroudy *et al.*, 1985) yielding cDNA probes for use in the detection of HAV-RNA by cDNA-RNA hybridization. Karaycannis *et al.* (1988) have reported a study in which they have used RIA and molecular hybridization to establish the presence of HAV and anti-HAV IgA (HAV immune complexes in serial stool specimens from the time of infection to recovery) in experimentally infected tamarins and to correlate them to biochemical and serological findings. HAV-RNA was detected by molecular hybridization in faecal specimens and tissues. HAV-RNA was present in the faeces within 24 hrs of inoculation and then increased in concentration until the 9th day when it continued through the acute disease into the early convalescence phase. Faecal anti-HAV IgA appeared within one week of infection and immune complexes cotaining the virus were found to be present until the early convalescent period.

In another report (Prévot and Kopecka, 1988), two independent methods for HAV detection were used: enzyme-linked immunosorbent assay (ELISA) using polyclonal and

monoclonal antibodies and molecular hybridization using subgenomic cRNA transcripts (RIBOPROBES) of HAV cDNA. Both methods reportedly permitted the detection of HAV replication in two different cell lines (PLC/PRF5 and VC-10).

In yet another study Shieh *et al.* (1991) have used ssRNA probes to detect HAV. Clone of cDNA encoding the 5'-most 1 kb of the HAV and coxsackievirus B3 genomes were subcloned into T7/SP6 RNA transcription vectors.

Progress in the diagnosis of HBV infection

Since the discovery of reproducible specific serologic markers in type B viral hepatitis, initiated more than two decades ago (Blumberg *et al.*, 1965, 1967; Prince, 1968), substantial progress has been achieved concerning the virus-related markers and immune response during infection and hence towards prevention of this widespread form of hepatitis. Many of the markers and the methods for their detection have now become recognized as standard diagnostic tools (Kurstak, 1993).

HBV serology has become extremely refined. Over the years, several serologic markers in the various courses of HBV infection have evolved. As well as the recognized hepatitis B surface (HBs), core (HBc), and e (HBe) antigen-antibody new markers have been introduced including pre-S1 and pre-S2 for the enveloping and the functional proteins.

New automates have been introduced allowing flexibility in the different tests in accordance to precise needs. The monitoring of the pre-S1 antigen provides a relevant correlate of viral replication. Molecular hybridization techniques have provided a sensitive and specific tool for the detection of HBV DNA sequences, particularly in serum, which in turn has proved to be a valuable additional marker of active viral replication in HBV infection. Employment of HBV DNA analysis has extensively improved the understanding of the natural history of HBV infection and the complex clinical pattern of hepatitis B. Studies using HBV DNA analysis have in addition, pointed to intriguing interactions between other viral diseases and HBV infection. The quantitative determination of HBV DNA, pre-S1 Ag and anti-HBc IgM seem most useful for the decision to use, and for the monitoring of antiviral treatment.

Detection of HBsAg and of anti-HBs

Currently, the optimal techniques for HBsAg detection appear to be highly sensitive, specific and rapid third generation tests. ELISA, EIA, RIA and reverse passive haemagglutination (RPHA) methods are commercially available, simple, rapidly performed, reproducible and very sensitive. These techniques have found extensive use in screening blood donors for HBsAg (Seidi and Trautman, 1981). Despite the proven superiority of RIA for HBsAg detection by blood bank, the limited dating period of RIA testing kits and the problems in handling radioisotopes have prompted many laboratories to adopt EIA procedures which obviate the problems of RIA testing and provide the third generation sensitivity.

Recently, a method for determining levels of serum HBsAg have been developed by Yamashiki *et al.* (1993) applying the principles of the integrating sphere turbidimetric assay (ISTA). Using this method, the minimum detectable level of HBsAg is 15 ng/ml, i.e., ISTA is three times more sensitive than the RPHA method. Reproducibility and specificity appear to be excellent with ISTA. Serum HBsAg could be reliably measured with ISTA by the utilization of a fully automated EL-1000 analyzer. This rapid and simple method seems to be promising in clinical usage.

At present, RIA is the most widely used test for the anti-HBs detection, particularly, due to their commercial availability, high sensitivity and specificity. Recently Jackson *et al.* (1988) have reported the development of a solid-phase enzyme-labelled immunoassay that detects antibody to HBsAg. Microwells coated with HBsAg are incubated with test sample and binding to the solid phase of any specific antibody is identified with the use of HBsAg conjugated with alkaline phosphatase. A novel enzyme cyclic system is used to amplify the activity of the bound label enzyme, thereby producing an assay quicker to perform and more sensitive than conventional label enzyme systems. The degree of concurrence with anti-HBc testing in evaluation of specificity of the assay suggested that the assay be more specific than an existing commercial anti-HBs RIA.

Detection of HBcAg and anti-HBc

Tests reported to detect HBcAg include rather sophisticated methods as immunoelectron microscopy (IEM), RIA, immunoperoxidase (IP) and immunofluorescence (IF). Tests detect HBcAg, however, are restricted to studies of liver biopsy materials. HBcAg particles are generally purified from infected liver cells, and sera of chronic hepatitis B patients after detergent treatment to release HBcAg from within HBV. Tests for HBcAg seem to be rarely used in the diagnosis of hepatitis B since this internal antigen is absent in serum. The presence of HBcAg in infected liver cells has been assumed to indicate ongoing virus replication.

Tests to detect anti-HBc over the years included counterelectrophoresis (CEP), complement fixation (CF), RIA and EIA. Measurement of anti-HBc IgM could effectively differentiate chronic disease states from acute hepatitis B cases. Recombinant DNA techniques have enabled the syn-

thesis of HBcAg in *Escherichia coli* and thereby obviating the problem of severity of HBcAg, traditionally isolated from autopsy livers. Scorer and Cayzer (1988) have reportedly evaluated a new ELISA test using directly labelled HBcAg as specific for anti-HBc IgM detectable in 99% of sera from patients with acute hepatitis B. Commercial ELISA kits for the detection of anti-HBc (Corzyme, Abbott Laboratories) and anti-HBc IgM (Corzyme-M, Abbott Laboratories) are also currently available.

Detection of HBeAg and anti-HBe

HBeAg is useful indicator of increased infectivity due to association of HBeAg with HBV DNA polymerase, active viral replication and circulating HBV infection particles in serum. Anti-HBe is indicative of a reduced level of virus infectivity due to a decreased viral replication.

Sophisticated assays as RIA and EIA with more than a 1000-fold improved sensitivity over traditional haemagglutination method (Takahashi *et al.*, 1977) remain the mainstay for detection of HBeAg. The Abbott HBe-EIA system is utilized currently in many diagnostic laboratories. Recently, Robinson *et al.* (1992) have reported the development of fully automated microparticle EIA for the detection of HBeAg (IMxHBe) and anti-HBe (IMx anti-HBe), respectively. The result agreement between IMxHBe and Abbott HBe RIA/EIA was reportedly 99.7%, and that between IMx anti-HBe and anti-HBe RIA/EIA 95.8%. The IMx anti-HBe assay was 2- to 4-fold more sensitive than the current RIA as determined by serial dilution of anti-HBe reactive specimen. IMx anti-HBe may be useful in confirming prior exposure to HBV in blood screened positive by Corzyme.

New and useful markers of HBV infection

Pre-S1, pre-S2 and X are three additional coding regions leading to the expression of protein during the virus growth cycle which are extensively studied (Kurstak, 1993). The polymerase gene product apparently seems to have received least attention. In a study of renal dialysis patients, however, anti-polymerase antibodies have been reported as the earliest marker of HBV infection (Feitelson *et al.*, 1988). Both in human and experimental hepadnavirus infection in the woodchuck, anti-polymerase antibodies were found to be constant in fulminant hepatitis and common in acute HBV infection where they correlated directly with the existence of ongoing HBV infection. Interestingly enough, the occurence of anti-polymerase antibodies in chronic hepatitis B patients was about the same in those with HBeAg and those with anti-HBe. This finding lends support to the idea that ongoing viral replication in such patients may very well persist in the presence of anti-HBe.

Several groups of investigators have probed for HBxAg in tissues and for anti-HBx sera of patients with a variety of HBV-associated diseases (Levrero *et al.*, 1988; Vitvitski-Trepo *et al.*, 1990). The results obtained appear to be discrepant but may be attributable to different probes, either recombinant fusion proteins or synthetic peptides used. However, they are in agreement with the transactivation function of X protein. Expression of HBxAg in the liver has been confirmed universally. The detection of HBxAg on the plasma membrane of infected hepatocytes, together with a close correlation between HBxAg-positivity and raised serum alanine aminotransferase (ALT) indicates implication of HBxAg in the pathogenesis of HBV infection. The detection of HBxAg in the serum has been reported by Horike *et al.* (1991) and Feitelson *et al.* (1990). HBx antigenemia seems to be restricted mainly to chronic HBV infection with positivity for HBV DNA in serum HBcAg in liver tissue.

Anti-HBx is detectable earlier, and is found in both acute and chronic infection. Titers and prevalence appear to correlate with the intensity of HBV replication. Seroconversion of HBxAg to anti-HBx-positivity occurs in most patients before HBeAg to anti-HBe seroconversion (Trepo *et al.*, 1993). Pre-S antigen as a marker of viral replication has been evaluated and all studies seem to find much higher values of pre-S antigen in HBV DNA-positive cases as compared with negative ones with variation in the proportion of HBV-DNA cases reactive for pre-S antigen according to the technique used (Trepo *et al.*, 1993). A double RIA using specific monoclonal antibody recognizing aa 21-47 region of the pre-S1 antigen sequence on the envelope of HBV has been developed (Petit *et al.*, 1990). Using this assay, the expression of pre-S1 antigen correlated well with levels of HBV replication in patients with chronic active hepatitis B. Except the development of some prototypes commercial availability doesn't yet exist for any for these new markers (polymerase, HBxAg, anti-HBx, pre-S1 and pre-S2 antigens or antibodies).

Detection of HBV DNA and the role of the polymerase chain reaction (PCR) in the diagnosis of HBV infection

Molecular hybridization techniques have provided a sensitive and specific tool for the detection of HBV DNA sequences in serum which in turn has proved to be a valuable additional marker of active viral replication in HBV infection. Evaluation of HBV DNA levels appears to be of value in monitoring antiviral therapy and in the clinical assessment of chronic carriers (Schmilovitz-Weiss *et al.*, 1993; Kuhns *et al.*, 1988).

In the past, two methods were available for detecting the presence of HBV DNA in serum, the endogenous DNA polymerase reaction and dot or slot blot hybridization anal-

ysis (Lieberman *et al.*, 1983; Walter *et al.*, 1987). Of the two methods, slot blot hybridization analysis was demonstrated to be more sensitive. However, a new technique, PCR assay has been shown to detect 10 fg of HBV DNA (Kaneko *et al.*, 1989). In another study Yokosuka *et al.* (1991) demonstrated that HBV DNA could be detected at the level of one virion by PCR. The HBV DNA sequence was determined by direct sequencing in a matter od days. Shish *et al.* (1991) developed an HBV PCR DNA typing procedure to assess the value of classifying hepatitis B virus strains at the genome level with products from the PCR. The design of this procedure is based on the selective sensitivity of the PCR product to digestion with different restriction endonucleases and on size of the fragment resulting from a specific nuclease digestion. In another interesting study related to HBV DNA methodology, Manzin *et al.* (1991) have developed a rapid single-step procedure for the detection of HBV DNA sequences from serum samples by PCR. The novel technique appears to be efficient when compared to the standard proteinase K-phenol-chloroform method offering the advantage of being faster and easily adaptable to the routine processing of a high number of clinical samples by PCR and even by spot hybridization techniques. Using HBV DNA assay by PCR Gerken *et al.* (1991) concluded that in patients who became HBeAg-negative during antiviral treatment the viral DNA disappeared and that this assay is for more proficient than the dot blot method. With their PCR assay the authors were capable of detecting as few as 30 copies of serum viral DNA and 100 ag of cloned HBV DNA. In addition they demonstrated in patients with chronic hepatitis B undergoing interferon therapy that serum HBV DNA detected by PCR is more sensitive marker of HBV replication that pre-S and S proteins.

PCR has undoubtedly modified the approach to the diagnosis of viral hepatitis. The main advantages of PCR thus appear to be its extreme sensitivity and the possibility to develop rapid assays with the utilization of non-radioactive probes. Its usefulness has been documented by the demonstration of viraemia, the identification od seronegative viral carriers and studies on mother-to-child transmission of infection. While it provides a direct and highly sensitive identification of viral genome, it appears to have three important limitations to its routine use: (a) the risk of false positive results attributable to contamination, (b) difficulties in developing quantitative tests, and (c) the need for automation and use of non-radioactive probes for wider availability of this technique (Bréchot, 1993).

Although the reliability of hepatitis virus serology performed by clinical laboratories appears to be generally good or even excellent, internal and external quality controls would seem to be extremely useful as suggested recently (Gerken *et al.*, 1991; Kurstak, 1993; Kurstak *et al.*, 1995).

Current status of the diagnosis of HDV infection

The diagnosis of HDV infection as delta hepatitis is often difficult and the serological assay commercially available and in use is the RIA for anti-HDV. Testing for IgM to hepatitis B core antigen, the serological marker of acute hepatitis B, seems to provide a method for distinguishing co-infection from superinfection in cases of acute hepatitis D. In fact, it has been suggested that the best cost-effective procedure may be to test any patient with HBV markers for anti-HDV, 30-40 days after the onset of symptoms (Salassa *et al.*, 1991).

Progress in the molecular biology of HDV has made available new diagnostic assays which complement immunological assays for HDVAg and anti-HDV (Shattock and Morris, 1991). Hybridization assays available for the direct estimation of the HDV genome are based on probing sera with DNA clones complementary to HDV RNA (Smedile *et al.*, 1986) or with cDNA-derived RNA probe (Smedile *et al.*, 1987). Immuno (Western) blot analyses developed enable determination of hepatitis D antigenemia and in chronic hepatitis yield positive results in presence of anti-HDV. In general, assays utilizing RNA probes complementary to virion RNA appear to be rather more sensitive.

Various EIA procedures have been developed for notable detection of anti-HDV and in recent years for HDVAg itself. Among them is the EIA for HDVAg and anti-HDV from Organon Teknika, the Delta Assay for HDVAg from Noctech, and the Abbott anti-delta EIA, a test for anti-HDV from Abbott Laboratories. Matthyssen *et al.* (1988) have reported the development of a simple and easily performed EIA for the detection of anti-HDV with the use of the same reagents as for the direct test for HDVAg. The test is an EIA based on a competitive sandwich-inhibition principle and according to the authors the result could be obtained within 3 hrs as a distinct colour. The assay provides the potential for detection of both HDVAg and its antibody. The analytical sensitivity of this test for anti-HDV in human anti-HDV-positive sera was either equal to or better than the Abbott anti-delta EIA and reportedly had a clinical specificity of 99.9%. This new easily performed EIA for detection of anti-HDV with high specificity and acceptable sensitivity appears to be promising as well as attractive since the essential agents are readily available in a kit combination (Hepanostika anti-delta, Organon Teknika). An interesting study was performed by Shattock and Morris (1991) on evaluation of six commercial EIAs for detection of HDVAg, anti-HDV and anti-HDV IgM. They concluded that in detecting HDVAg, the Noctech, Pasteur and Welcome assays had 100% sensitivity; The Organon reagents gave 59.5% sensitivity without detergent and 64.3% with detergent. The Sorin assays gave 23.8% sensitivity. In detection of anti-HDV all six EIAs gave 97.8% to 100% sensitivity and re-

acted with all anti-HDV-positive specimen. In detection of anti-HDV IgM the Noetech, Sorin and Welcome assays had 100% sensitivity. Although there has been a substantial improvement of previously evaluated assays, major differences still remain with regard to sensitivity among some assays for HDVAg detection.

Molecular hybridization for HDV RNA is the newest assay in diagnosing acute HDV infection and substantiating the diagnosis of chronic disease (Smedile *et al.*, 1987). The hybridization assays currently available are directed towards the genome of HDV in serum appear to be the best means of monitoring HDV replication. Rasshafle *et al.* (1988) reported the use of a spot blot hybridization assay with a RNA probe derived from a 650 bp cDNA fragment cloned into plasmid Gemini 2. The authors characterized this assay as a very simple and sensitive method for detection of HDV RNA. However, in this study, despite the use of the RNA probe system with ^{32}P-labelled RNA, the most sensitive and specific probe for detection of minute amounts of RNA, only 13 (76%) of 17 patients with HDVAg in hepatocytes were indeed found to be positive for HDV RNA. A spot or dot blot hybridization procedure has been since developed (Guyta *et al.*, 1989) and tested with a new HDV cDNA probe. An excellent correlation between the spot and Northern blot hybridization was achieved and furthermore the method is reportedly very rapid, sensitive and specific.

The usefulness of PCR in HDV RNA determination has been studied (Madejon *et al.*, 1991). The results suggest that HDV RNA detection by gene amplification is 10,000 times more sensitive than the slot blot hybridization and allows the detection of viral replication in patients without other viral replication markers. Cariani *et al.* (1992) have reported the development of a non-radioactive assay of HDV RNA in serum by combining reverse transcription of RNA, PCR of the complementary DNA and EIA detection of the PCR products using a monoclonal antibody specific for dsDNA. This DNA EIA is reported to have a limit of detection of cloned HDV RNA similar to that of standard PCR followed by Southern blot and to be 10^3 to 10^4 times more sensitive than the direct dot blot hybridization. This DNA EIA may be a potentially useful method for therapeutic monitoring in chronic HDV infection and may even perhaps contribute to a wider application of PCR in clinical laboratory. The application of PCR in the direct diagnosis of HDV viremia has been also achieved in a recent study by Lecot *et al.* (1993).

Current knowledge of diagnosis of HEV infection

HEV seems to be responsible for most epidemics of non-A, non-B hepatitis in developing countries and affecting mainly young- to middle-aged adults (Zuckerman, 1990; Goldsmith *et al.*, 1992; Herrera, 1993; Kurstak, 1993).

The diagnosis of HEV infection has been based until now on the detection by IEM of virus-like particles of 27-34 nm in diameter in faecal specimens from infected patients serologically-negative to other viral hepatitides (Bradley *et al.*, 1987). Some progress was made towards a diagnostic test with the identification of common HEV epitopes (Yarbough *et al.*, 1991) with the development of an ELISA based on cloned recombinant HEVAg to detect anti-HEV IgM and IgG. Lok *et al.* (1992) demonstrated the validity of the recombinant-based immunoassays for the diagnosis of hepatitis E in a seroepidemiological survey in Hong Kong.

Recently Goldmsmith *et al.* (1992) diagnosed the HEV infection using the ELISA with four HEVAgs with anti-HEV IgG and IgM. The serum was considered reactive for anti-HEV when the ELISA signal to HEV-glutathione S-transferase fusion proteins was at least three times higher than that to non-recombinant glutathione S-transferase proteins. This ELISA detects both anti-HEV IgM and IgG and is recommended as a convenient method for the diagnosis of acute or past HEV infection. A newly developed Western blot assay for anti-HEV appears to be specific for the diagnosis of icteric non-A, non-B hepatitis (Tram *et al.*, 1991).

Recent strides in the diagnosis of HCV infections

The tremendous progress made towards elucidating the structure and the genome of the HCV isolated from human carriers has paved the way towards the development of diagnostic tools for HCV infections (Kurstak *et al.*, 1995).

Antibody assays

The molecular characterization of the HCV genome led to the development of immunoassays for the detection anti-HCV. The first generation EIA basically utilized a single recombinant viral protein (C100-3) for the diagnosis of HCV infection (Kuo *et al.*, 1989).

Use of the C100-3 did result in a significant assessment of the disease in transfusion-associated hepatitis infections (Donahue *et al.*, 1992). However, it now appears that the use of C100-3 EIA generated significant numbers of not only false-positive but also false-negative results (Okamoto *et al.*, 1991; Sugitani *et al.*, 1992). To circumvent such problems, second and third generation serological tests that include additional structural and non-structural proteins or peptide epitopes have currently been evaluated in screening as well as confirmatory assays (Boudart *et al.*, 1992; Okamoto *et al.*, 1992). These tests in similarity with the

first generation test rely on the detection of an antibody response to viral antigens. Okamoto *et al.* (1992) developed immunoassays to detect antibodies against oligopeptides deduced from the putative core gene of HCV and compared their performance with that of the commercial assay for antibodies against the product of non-structural regions of HCV (anti-C100-3). The results of their study seem to suggest that antibodies against antigenic determinants of the HCV core could possibly complement anti-C100-3 for the reliable diagnosis of non-A, non-B hepatitis.

Detection of HCV

In view of the variation of the period between infection and host-immune response fo different HCV antigens among individuals, it appears to be desirable to test directly for viral antigens, nucleic acids or even both. However, because of the low titers of circulating virus common in HCV infection, reliable detection of viral antigens remains as yet undocumented. Consequently, efforts have been directed mainly towards the detection of HCV RNA in clinical samples.

The utilization of PCR techniques to amplify reverse-transcribed cDNA permits a very sensitive assay for viral specimens. Despite the absolute necessity to perform negative control reactions to exclude cross contamination and performance of assays in triplicate, the ability to detect small number of viral RNA molecules by PCR assays represents an extremely sensitive assay for HCV infection. The optimal region for PCR assay now very likely seems to be the 5'-leader RNA, generally well-conserved among different HCV isolates. On the basis of a signature sequence for HCV (5'-untranslated region), Cha *et al.* (1991) reported the development of a viral RNA assay using cDNA synthesis with reverse transcriptase, followed by PCR. This new assay was compared with the Ortho-Chiron C100-3 HCV ELISA. On the basis of the results obtained the authors suggest that the use of probes and primers from the 5'-PUT region (as opposed to primers from other regions) should not lead to false-negative results due to nucleic acid sequence variations.

The fact that most PCR assays have qualitative limits, their use is suggested to applications where only the presence or absence of target nucleic acids is to be determined. Hence, establishment of calibration of an external standard, identical to target sequences or comparison with co-amplified, unrelated "reporter" RNA appears to be a necessity for quantitation of target sequences using PCR technique. Interestingly enough, Lundeberg *et al.* (1991) have reported the demonstration of the quantitation of the amount of target DNA/RNA by competitive PCR. Kanoko *et al.* (1992) successfully utilized this competitive PCR to quantitative HCV RNA in sera of patients with chronic hepatitis. Quantitation of HCV RNA carrier the potential for evaliating treatment of HCV infection.

Much effort has been devoted to the detection of HCV RNA in clinical samples with a combination of reverse transcription and PCR (RT-PCR). Extremely sensitive PCR-based protocols are now known and proven to be useful in confirming and monitoring HCV infections (Sugitani *et al.*, 1992). However, as apparent from the recent quality control study of Zaaijer *et al.* (1991), reports of the presence of HCV should be interpreted with care until reliable HCV RNA becomes widely available.

In an effort towards achieving an increase in the sensitivity and reliability of PCR analysis, a nested PCR has been exploited (Garson *et al.*, 1990). A pitfall of nested PCR appears to be that the second PCR cycle is set-up the presence of first PCR cycle amplification products thereby increasing the risk of contamination by product DNA. As an alternative approach to nested PCR, an one-stage PCR amplification combined with Southern blot analysis (Han *et al.*, 1991) or liquid hybridization with radioactive oligonucleotide probes (Gretch *et al.*, 1992) has been used. Recently however, Gretch *et al.* (1993) have described an one-stage PCR assay of HCV RNA which when combined with either liquid hybridization or Southern blot analysis has been noted to be equal in sensitivity to the nested PCR. Furthermore, this study also established that one cycle of PCR plus liquid hybridization offers sufficient sensitivity as well as less potential for contamination.

To tackle the problem of contamination of secondary amplification with products acumulated from previous amplifications a method for heminested PCR using drop-in/drop-out primers was recently developed (Ehrlich *et al.*, 1991). This method is accomplishable in a single step. Romeo *et al.* (1993) have utilized this novel RT-PCR assay for the analysis of the prevalence of HCV complementary RNA in a set of 53 plasma specimens from blood donations that were repeatedly reactive for anti-HCV with the first generation EIA. Of 21 specimens that were also reactive for anti-HCV in a four-antigen recombinant immunoblot assay (RIBA-2) 20 (95%) contained detectable levels of HCV RNA. With the future development of reliable automated RT-PCR assays it appears that the detection of HCV RNA by RT-PCR does provide practical confirmation test of particular importance in the clinical management of apparent healthy blood donors who demonstrate anti-HCV in supplementary RIBA testing.

CONCLUSION

The effective use of new molecular biological techniques towards the reliable diagnosis of HCV and other viral liver infections has been updated in this review. The applications

of PCR tecniques with amplification of reverse transcribed cDNA seems to provide an effective means for assaying HCV infections. A very recent one-stage PCR assay of HCV RNA combined with either liquid hybridization or Southern blot analysis, equal in sensitivity to the nested PCR assay but with sharply reduced potential for contamination appears to be promising. Future further development of reliable and automated RT-PCR assay would be particularly interesting for the diagnosis of HCV infections.

PCR apparently remains the most useful test for the appraisal of HBV infection in sero-negative patients with liver disease. It has now made possible the confirmation of observations made with the spot blot or Southern blot test and provided access to the nucleotide sequence analysis of these viral mutant forms. The rapidity and simplicity of the newly developed latex agglutination method using ISTA also makes it a viable alternative for the determination of HBsAg. Cloning of HEV, sequencing of the viral genome and expression of recombinant HEV proteins has undoubtedly facilitated significant progress in the development of methods for identification of HEV infection in patients. Recently the availability of specific, more sensitive assays as recombinant-based EIAs has made the diagnosis of hepatitis E very much practicable.

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