Approaches of the Diagnosis of Hepatitis Viruses

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Abstract

Hepatitis virus infection occurs in close to a billion people worldwide at some point in their lifetimes. Hepatitis B and C viruses together account for infections in half a billion people and are considered the most carcinogenic of any known biological agent. The diagnostic approaches to detect hepatitis viruses are discussed.

Index Entries: Hepatitis virus, diagnosis; hepatitis viruses; RT-PCR; PCR.

1. Introduction

Acute and chronic liver disease is most commonly caused by hepatitis viruses. Liver diseases resulting from hepatitis viruses share the common characteristic of causing inflammation of the liver. Hepatitis viruses affect a significant population in the world and is a serious public health concern requiring considerable effort to ensure that the blood, water, and food supply remains free of these viruses. The early discovery of hepatitis viruses began in the mid-1960s with the discovery of hepatitis B virus (HBV) and continued in the 1970s with the discovery of hepatitis A (HAV) and D (∂) (HDV) viruses. Two other major causative agents of viral hepatitis were recognized and referred to as non-A, non-B hepatitis viruses (NANBH) for almost two decades. Advances in molecular techniques in the late 1980s, especially the polymerase chain reaction (PCR), have ushered in the discovery of two new viruses, hepatitis C (HCV) and Hepatitis E (HEV) viruses earlier referred to as NANBH. Thus, the most common causes of viral hepatitis are hepatitis A, B, C, D, and E ***(1)***. Recently, the discovery of three other hepatitis viruses, two of which are referred to as GB-A and GB-B ***(2)*** and a third as GB-C, has been reported. All are closely related to the flavivirus family. It remains to be seen how many more will be discovered; there is a general belief at this time that Genelabs, Inc. (Redwood City, CA) has isolated a new hepatitis virus called hepatitis G virus, but there is uncertainty as to the significance of the recently discovered and less prevalent viruses. The focus of this article therefore is the more common hepatitis viruses. It needs to be emphasized that there are three other pathogenic viruses, Epstein-Barr virus (EBV), cytomegalovirus (CMV), and enterovirus, that are capable of causing inflammation of the liver, but as they are not primarily hepatotropic, diagnostic protocols for these viruses are not discussed here. A schematic of the major and minor causes of viral hepatitis and the primary routes of transmission of the major hepatitis viruses is illustrated in **Fig. 1**.

Since the diagnosis of hepatitis virus infection is also a commercial business, I feel compelled to add the following qualifier. Several protocols are discussed in this chapter, some of which require the use of commercial kits, and some of which can be performed by purchasing individual items. Every effort has been made to provide the information that was published as well as submitted by companies, and to include all the available options for the diagnosis and monitoring of viral hepatitis infection without endorsing or recommending any particular product. Also, the exclusion of any

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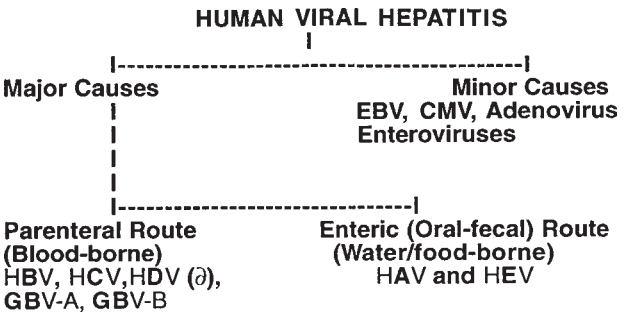


Fig. 1. Schematic of the major and minor causes of viral hepatitis and the primary routes of transmission of the major hepatitis viruses.

procedure or product/kit should not be taken as an indication of not being suitable for substitution of the ones that are described here.

1.1. Major Hepatitis Viruses A, B, C, D, and E

Before proceeding to performing the diagnostic protocol, it is imperative to possess a certain basic understanding of the major hepatitis viruses. These viruses are one of the most serious biohazard-ous occupational risks, and any handling of specimens should be done in places of restricted admittance by persons trained in the proper handling and disposal of biohazardous material. The route of transmission, the period of incubation, the vaccine administered in the person being tested, and the treatment that the person is undergoing have an important bearing on the appearance of the molecule being tested in the material being tested. Therefore, information summarizing the classification, mode of transmission, period of incubation, preventive intervention, prophylaxis, and treatment of major hepatitis viruses is given in **Table 1**.

The HAV genomic organization, and primers for reverse transcription-PCR (RT-PCR) ***(3–5)***, and antigens for enzyme-linked immunoabsorbent assay (ELISA) ***(6)*** are shown in **Fig. 2**. HAV-specific IgM antibodies are present in serum by the time symptoms of acute hepatitis appear ***(6)***. In cases in which symptoms appear a few days prior to the appearance of IgM, a second sample should be drawn after 1–2 wk. HAV-RNA appears as early as 2 d postinfection ***(7)*** and the amount of RNA as an indication of viremia is directly proportional to the IgM titers. Serum IgG is indicative of exposure and persists for long periods. Kits to assay the presence of antibodies against HAV can be obtained from Abbott Laboratories (North Chicago, IL). Both kits use inactivated virus as the antigen, making it difficult to prepare antigens. The sequence of the most antigenic region of the HAV capsid, derived by computer analysis using the MacVector program (Oxford Molecular Group, Campbell, CA), is provided for those willing to attempt to set up their own ELISA. Use of RT-PCR can allow the detection of RNA isolated from stools and can confirm diagnosis. Methods for quantitation of HAV by PCR have been described recently ***(4)***.

The genomic organization of HBV and the primers used for detection of the HBV-DNA in serum or plasma are shown in **Fig. 3**. Also shown are the open reading frames for the different proteins of HBV. The presence of HBV surface antigen (HBsAg) and "e" antigen (HBeAg) is indicative of active viral replication during the acute phase in most patients and it usually suggests severe liver damage. An accurate and sensitive reflection of viral replication in the liver is the presence of HBV-DNA in serum ***(8)***. Absence of HBV-DNA in serum, but presence in liver tissue could be an indication of integration in the genome. HBV-DNA is present in serum in 50% of patients with fulminant atypical HCV infection. The presence of antibodies to HBVSAg is indicative of recovery and immunity or a successful immune response to vaccination. High IgM antibody titers against the core protein are indicative of active infection, while low titers are indicative of chronic persistent infection. The response to interferon treatment results in lowering of serum HBV-DNA, and it is highly recommended that those patients undergoing interferon treatment be monitored by a quantitative HBV-DNA procedure to determine the response.

The genomic organization of HCV and the primers used for detection of the HCV-RNA in serum or plasma are shown in **Fig. 4**. Also shown are the open reading frames for the different proteins of HCV and the antigenic regions used to

Table 1
Characterization of Major Hepatitis Viruses

Virus characteristics	HAV	HBV	HCV	HDV	HEV
Virion size	27nm	42nm	30–50nm	35 nm	37–34 nm
Nucleic acid (genome)	ssRNA	dsDNA	ssRNA	RNA	RNA
Family	Picornaviridae	Hepadnaviridae	Flavivridae	unclassified	Caliciviridae
Envelope	No Yes (HBsAg)	Yes (E1/E2)	Yes (HBsAg)	No	
Stability	Heat & acid stable	Acid sensitive	Ether sensitive	Acid sensitive	Stability (?)
Clinical and Epidemiologic features					
Incubation period	15–45 d	45–150 d	15–160 d	21–90 d	20–40 d
Onset	Abrupt	Abrupt	Insidious	Insidious	Abrupt
Transmission	Fecal-oral, water, food, parenteral (rare)	Parenteral, sexual, perinatal	Parenteral (common) sexual and perinatal (possible)	Parenteral and sexual (common) perinatal (possible)	Fecal-oral, water, and food
Symptoms					
Jaundice	Children: 10% Adults: 70–80%	25%	25%	Varies	Varies
Asymptomatic	Most children	Most children Adults: 50%	About 75%	Rare	Rare
Presence of virus	Feces, blood, urine	Feces, blood, urine	Body fluids	Body fluids	Feces, blood, urine,
Severity/fatality	Mild (0.6% fatality)	Severe (1.4%)	Moderate (1–2%)	Often severe (30%)	0.5–3%; 10–20% in pregnant women
Prognosis	Generally good	Worse with age	Moderate	Worse with age	Good
Progression	Complete recovery	Cirrhosis, HCC	Cirrhosis, HCC	Unknown	Complete recovery
Chronic state	None	Adults: 6–10% Children: 25–50% Infants: 70–90%	50%	10–15%	None
Diagnosis	EIA, RIA, of IgM, RT-PCR	HBsAG, anti-HBe/c/s, bDNA, PCR	EIA, RIBA II, bDNA, RT-PCR	RT-PCR, Delta antigen/Ab	EIA IEM, RT-PCR
Immunization	Inactivated, IgG	rHBsAg, HBIG		rHBsAG	None
Therapy		rIFN-α	rIFN-α		

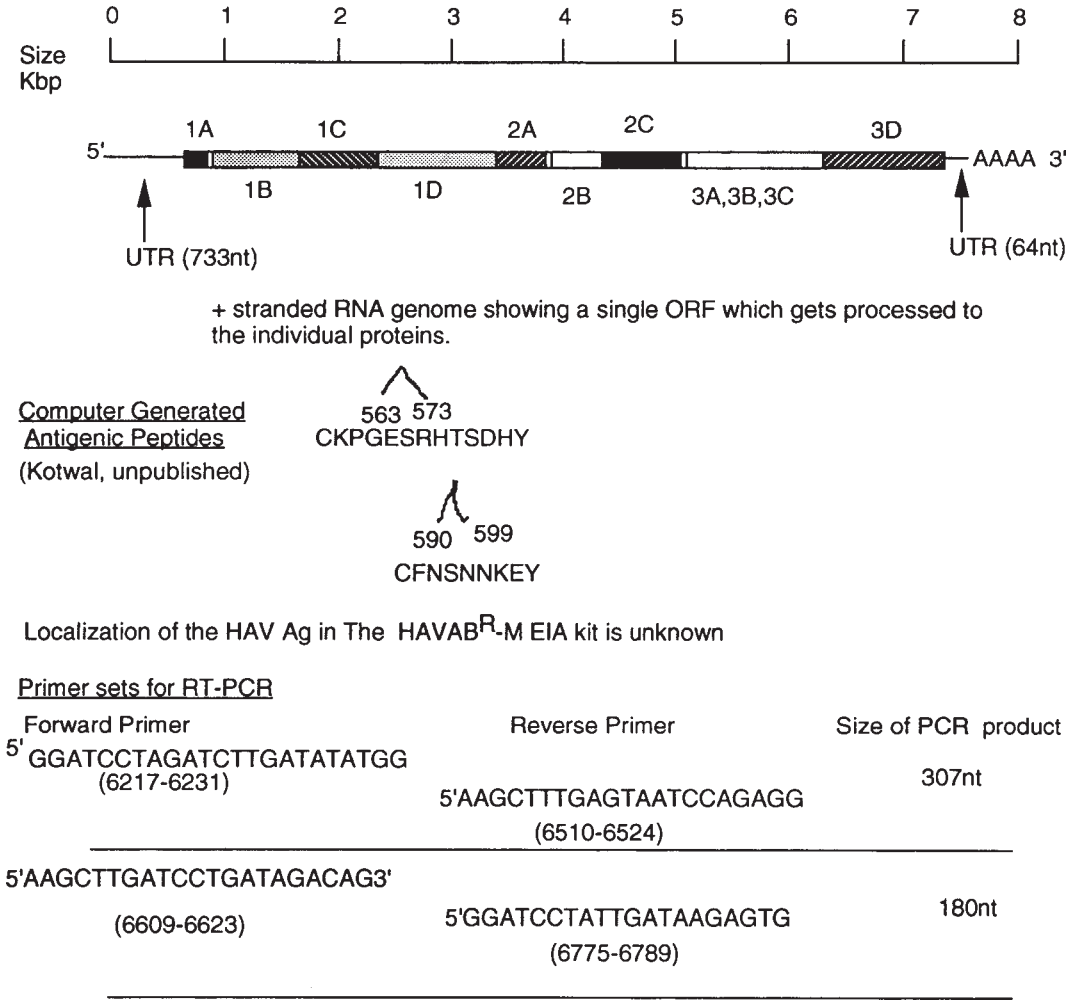


Fig. 2. Hepatitis A virus (HAV). Genome organization, location of individual proteins, antigenic regions, and primers for RT-PCR.

detect the antibody. Characterization of nucleotide sequences of HCV isolates from around the globe indicate a sequence conservation within the 5'-noncoding region and in and around the region encoding the putative RNA-binding nucleocapsid protein ***(9–15)***. These conserved regions have resulted in the development of new diagnostic assays for detecting HCV-specific antibodies ***(17–19)*** and for the detection of HCV-RNA by RT-PCR ***(20,21)***. The new assays have overcome the high false-positive rate and have greatly enhanced the chances of detecting HCV antibodies within the acute phase of infection ***(22,23)***. Additionally, direct detection of viral presence has become possible by setting up an ELISA to detect HCV-specific antigens ***(24,25)***. Most of the current assays measure only the IgG class, although one assay measures both IgG and IgM ***(17)***. This is achieved by the application of a mixture of the two secondary antibodies. Recent studies have shown that a transient IgM response can be detected concurrent with or earlier than IgG ***(26)***. A four-antigen recombinant immunoblot assay (RIBA) serves as a confirmatory test. The use of RT-PCR in the diagnosis of HCV infection or its confirmation is becoming common. The detection of HBV-DNA and HCV-RNA in the same sample has now become possible for those

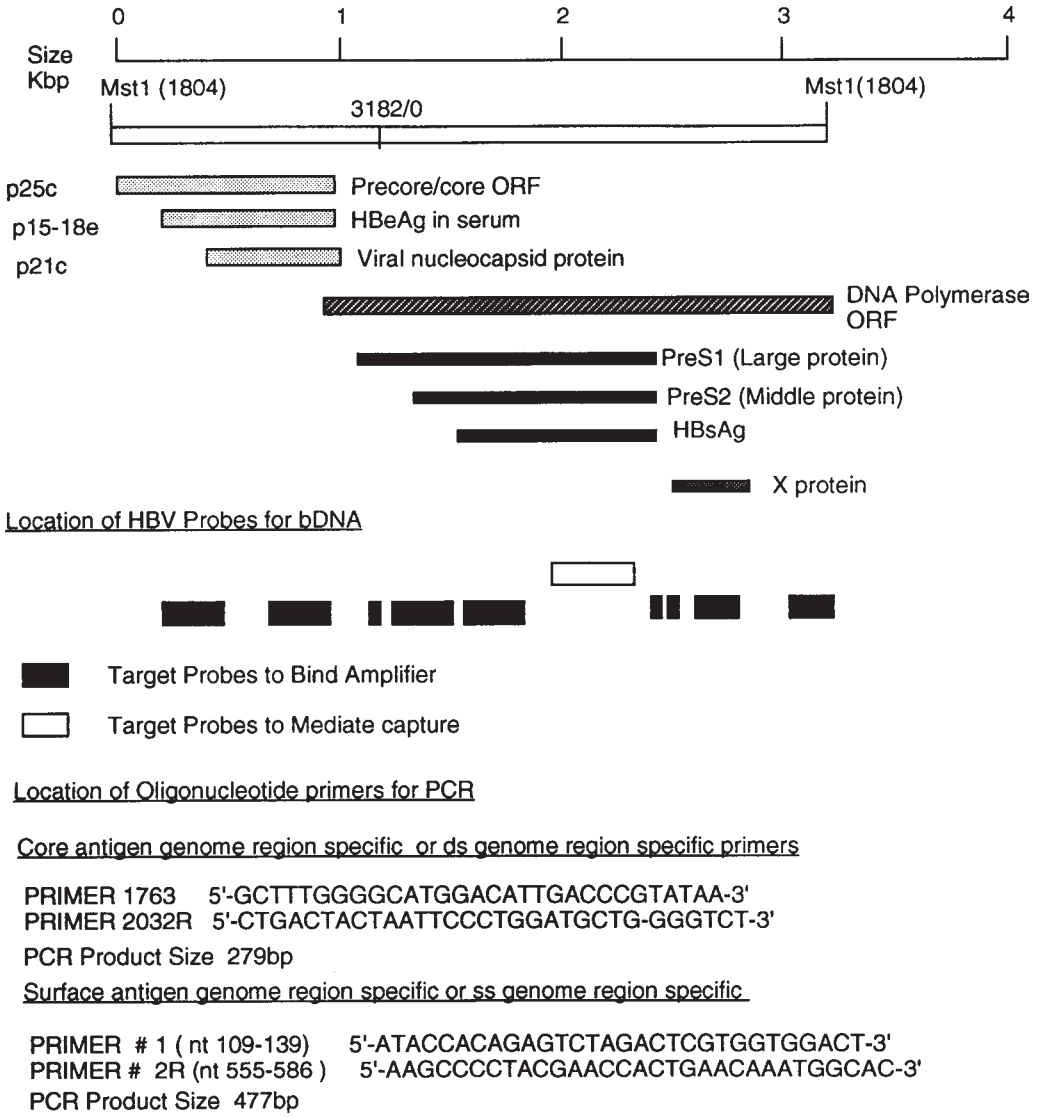
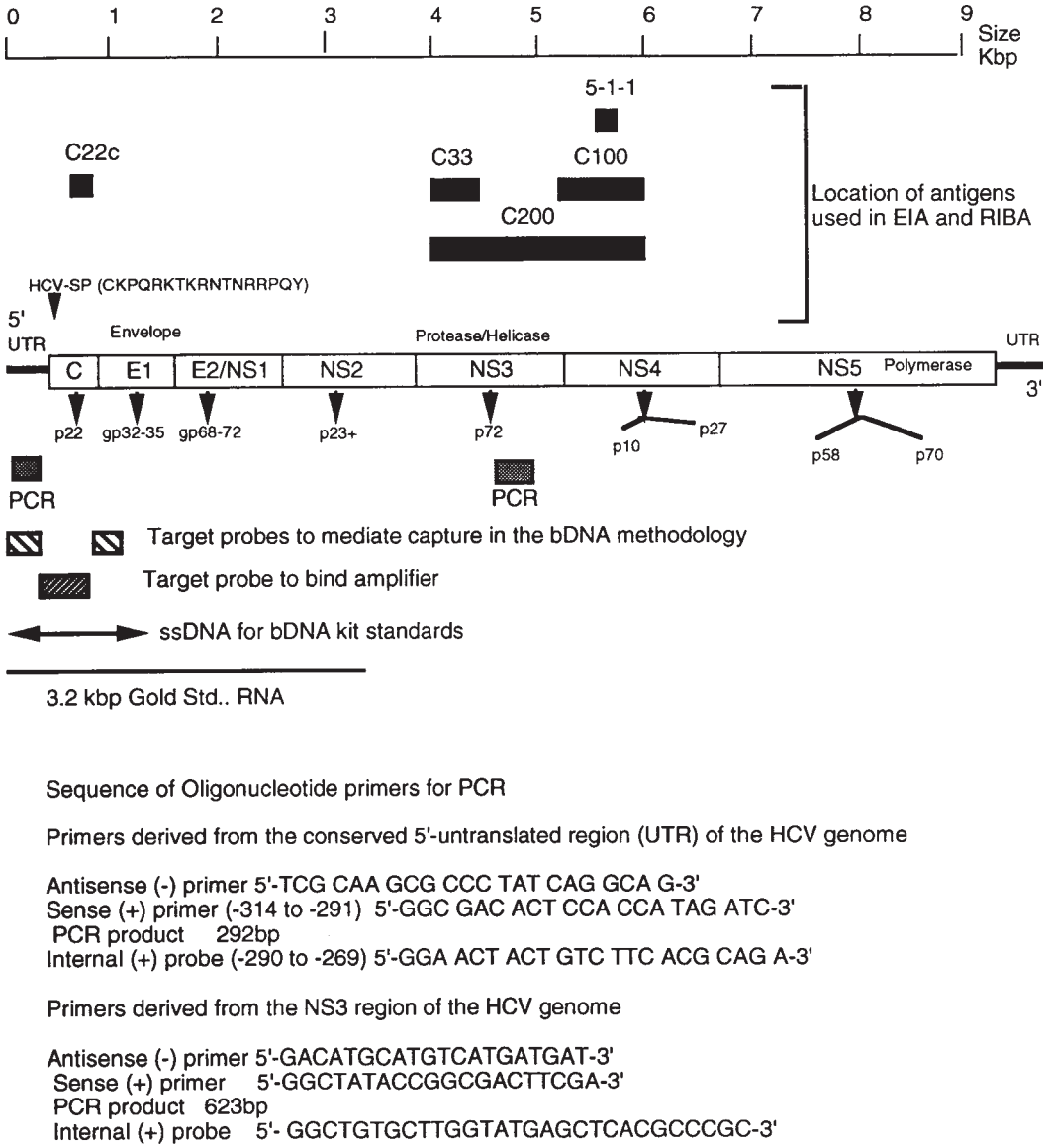


Fig. 3. Hepatitis B virus (HBV). Genome organization, location of individual proteins, probes for bDNA, and primers for PCR.

labs interested in minimizing costs and procedures *(27)*. The need for quantitative estimation of RNA levels to monitor patients' response to treatment either with interferon or other agents has resulted in the introduction of two new commercial assays, the branched DNA procedure *(28)* and the quantitative PCR method *(29)*. In addition, the ribonuclease protection assay can be set up from available individual reagents *(30)*. Genotyping of the HCV causing hepatitis, may become increasingly essential to perform, because of the predictive value of the genotype of the HCV found in patients and the severity of infection as well as the probability of favorable response to treatment with a given antiviral agent. There are to date 14 recognized genotypes, six of which are major genetic groups, based on the sequence analysis of the core gene from several isolates *(31)*.

HDV is a defective virus requiring HBsAg to form infectious virion particles and cause hepatitis *(32)*. Therefore, it can only cause severe hepa-



Sequence of Oligonucleotide primers for PCR

Primers derived from the conserved 5'-untranslated region (UTR) of the HCV genome

Antisense (-) primer 5'-TCG CAA GCG CCC TAT CAG GCA G-3'
Sense (+) primer (-314 to -291) 5'-GGC GAC ACT CCA CCA TAG ATC-3'
PCR product 292bp
Internal (+) probe (-290 to -269) 5'-GGA ACT ACT GTC TTC ACG CAG A-3'

Primers derived from the NS3 region of the HCV genome

Antisense (-) primer 5'-GACATGCATGTCATGATGAT-3'
Sense (+) primer 5'-GGCTATACCGGCGACTTCGA-3'
PCR product 623bp
Internal (+) probe 5'- GGCTGTGCTTGGTATGAGCTCACGCCCGC-3'

Fig. 4. Hepatitis C virus (HCV). Genome organization, location of individual proteins, probes for bDNA, and primers for PCR.

titis if it is acquired as a coinfection with HBV or as a superinfection in persons with chronic HBV. The genome of HDV is a single-stranded RNA that forms a structure similar to plant viroids owing to several base pairs that span the length of the RNA *(32)*. The antigenomic strand encodes a single antigenic nucleocapsid protein referred to as the HDV-specific antigen (HDAg), which functions in RNA packaging and replication. The HDAg consists of two forms, HDAg-L, a 27 kDa large form (215 amino acids), and HDAg-S, a 24 kDa small form (195 amino acids) *(32)*. The use of an immunoblot to ascertain the presence of HDAg is a very reliable and sensitive assay for active viral infection *(33)*. The IgG antibodies to the HDAg can be determined by ELISA, but the appearance of the IgG antibodies normally occurs after the loss of HDAg. Presence of the IgM antibodies to the HDAg may have to be determined by ELISA,

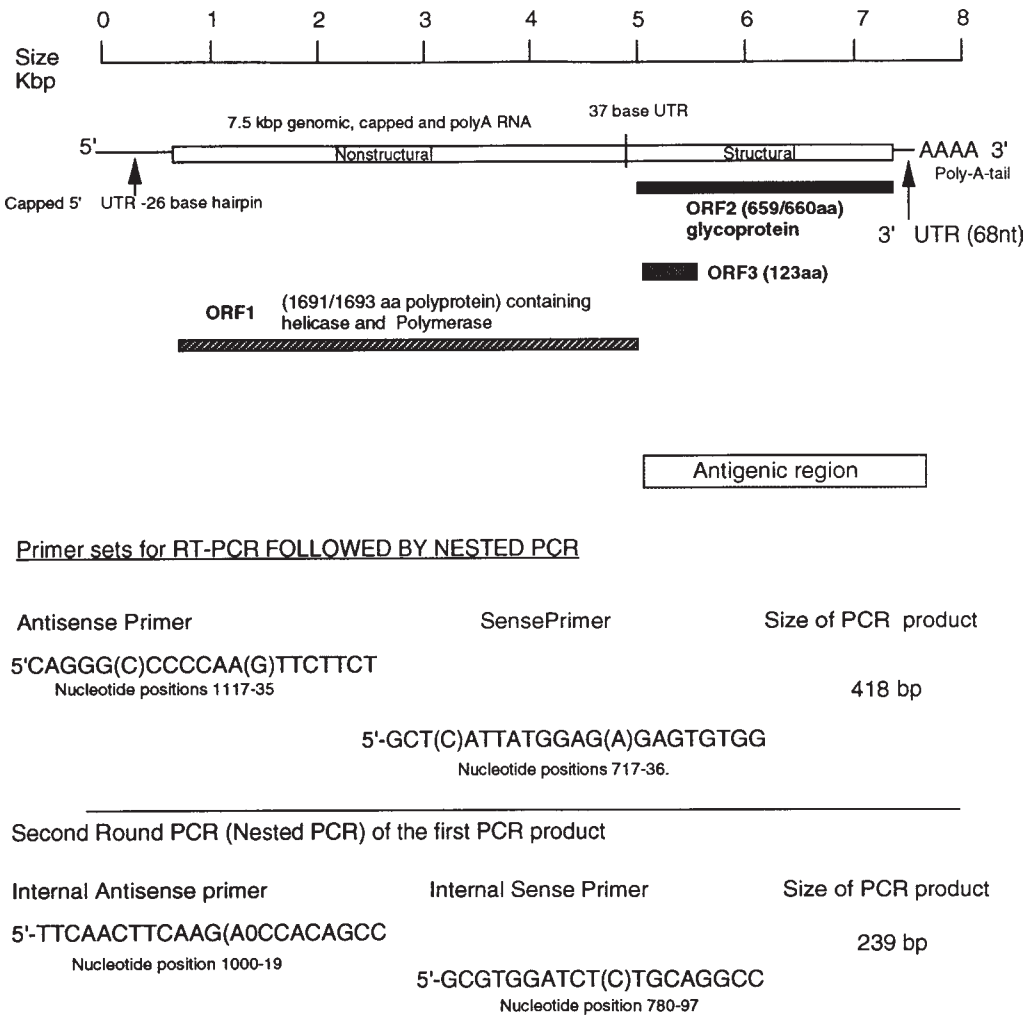


Fig. 5. Genome organization, location of individual proteins, antigenic regions, and primers for RT-PCR.

since they appear prior to the IgG and may overlap with the period when HDAg is still present. The detection of HDV-RNA by RT-PCR greatly facilitates the sensitive detection of the genomic RNA *(34)*. The antisense primer for such an RT-PCR would be AD2 (5'-ATGAGCCGGTCCGAGTCGAGGAAG-3') and the sense primer would be SD1 (5' TCACTGGG GTCGACAACTCTGGGG-AGA-3'). An internal primer D3 (5' CGAACGGACCAGATGGAGG TAGAC-TCCGGACCTAGGAAGAG-3') can be end-labeled and used as a probe to confirm the PCR product.

HEV genome organization and location of the antigenic regions *(35)* are shown in **Fig. 5**. The HEV-RNA can be isolated from stools and with the primers described above for HDV-RNA, an RT-PCR can be performed to determine the presence of the virus during the acute phase of the disease *(36)*. The antibody response to the virus that is elicited by the patients can be measured using an ELISA *(37)* or western blot *(38)*. It should be noted that by the time the antibodies appear, the patient will have recovered from the acute phase and therefore attempts must be made to perform the RT-PCR. If RT-PCR is unavailable, the testing for the antibody must be repeated within 2 wk if the earlier sample was negative. Because of the high mortality rate (20–30%) resulting from HEV acquired during pregnancy, it is critical that

RT-PCR be performed as early as possible after the first symptoms appear or if exposure is suspected.

The protocols for RNA isolation are based on the assumption that at the molecular level, RNAs from different viruses have similar properties to one another and that one isolation procedure can result in the extraction of RNA of similar purity from any virus. With respect to RT-PCR, although the conditions differ (but not significantly), optimization of conditions should always be done. Optimal conditions for each of the four RNA viruses are provided, but they should be standardized by the individual using the procedure.

1.2. Collection, Storage, and Processing of Specimen

The success and reproducibility of the protocols described is heavily dependent on the quality of the specimen, which in turn is dependent on the collection procedure, processing of the specimen and its storage.

The most commonly used specimen for diagnostic virology is serum. Most procedures for detection of antibody are performed using serum. In addition, the Quantiplex bDNA assay (Chiros Corp. Emeryville, CA) is performed using serum. Serum is the cell-free and fibrin-free portion of the blood and is obtained by allowing blood to clot. Plasma is the cell-free portion of blood obtained by centrifuging containing anticoagulant blood and siphoning off the supernatant. It has fibrin in it and is the preferred specimen for PCR and RT-PCR.

1.2.1. Serum Collection

Blood is drawn into a test tube without any anticoagulant, and the blood is allowed to clot at room temperature for no more than 1 h and either processed immediately as described or placed at 4°C or in wet ice for 2–4 h. Longer periods of waiting may cause degradation of the RNA. The serum is removed and placed in a sterile capped microfuge tube and the tube is spun at 12,000*g* for 5 min at 4°C. The serum is then removed without disturbing the pellet and aliquoted as desired; the aliquots are then placed in an ultralow temperature freezer at –80°C or lower. Repeated freezing and thawing may affect the quality of the specimen and should be avoided. Any transportation of serum samples should be done on dry ice. Serum that is to be used exclusively for antibody detection in an ELISA can be treated with 0.5% Nonidet P-40 (NP40) to give a final concentration of 0.1% NP40 and centrifuged at 10,000*g* for 5 min. The clear liquid between the pellet and the cloudy lipid layer on top is carefully removed and used in protocols for detection of antibody.

1.2.2. Plasma Collection

Blood is drawn into a test tube containing ethylenediaminetetraacetic acid (EDTA), mixed by inverting the tube 3–4 times, and placed immediately in a refrigerator at 4°C or on wet ice. Within 1–2 h, the blood is transferred to a centrifuge tube and centrifuged at 2000*g* for 10 min. The supernatant, or plasma, is carefully removed using a sterile plastic pipet, and transferred to several tubes, and stored at ultralow temperature (–80°C). **Caution:** Blood that has been frozen prior to separation cannot be used for obtaining plasma or serum, since the blood cells lyse, with only one cycle of freezing and thawing, and release their contents, such as, nucleases and proteases, which will affect the outcome of the experiment.

1.2.3. Water Collection

Since contaminated water is the most likely cause of transmission of HAV and HEV, it may be necessary from time to time to test the drinking water supply for the presence of these agents. In all likelihood even though the water used may be a source of the viral spread, it is likely to contain only a few virus particles in a fairly large amount of water. Therefore, the first step is to concentrate the water at least 100-fold before proceeding with any of the procedures described here. Concentration can be easily achieved by using ultrafilters such as the Amicon (Beverly, MA) stirred cell filters with 100,000 cutoff membranes under 70 psi N_2 in a cold room adjusted to 4°C. An ideal volume of preclaried water (achieved by centrifugation at 3000*g* for 10 min) to start with would be 500 mL and concentrated down to 5 mL. The concentrate can then be divided into aliquot and stored at –90°C.

1.2.4. Stool/Feces

One gram of stool sample is vigorously suspended (if the stool is hard this may require a homogenizer) in 10 mL of phosphate-buffered saline (PBS) or physiological saline in a sterile conical polypropylene or polystyrene tube and centrifuged at 3000*g* for 15 min at 4°C. The supernatant is carefully removed and passed through a bacteria-proof membrane (Millipore, Bedford, MA) filter (0.45 μm pore size), and the filtrate is concentrated about 25-fold using a centricon-10 filter (Amicon). The retentate is then stored at –90°C or processed for RNA extraction as described below (*see* **Subheading 3.1.1.**).

1.2.5. Liver

Liver removed during transplantation is cut into strips about one-half-inch wide, snap-frozen directly in liquid nitrogen, and stored at –70°C or below. Liver biopsy specimens are similarly frozen and stored.

1.2.6. Other Body Fluids

Body fluids other than plasma and serum may require testing. Such fluids should be placed in empty sterile tubes and quickly frozen and stored at ultra-low temperatures (–90°C).

1.2.7. Perpheral Blood Lymphocytes (PBLs)

Five milliliters of whole blood containing an anticoagulent agent such as, heparin or EDTA, stored at room temperature for no longer than 2 h is carefully overlayed on top of an equal volume of sterile Histopaque 1077 (Sigma, St. Louis, MO) in a sterile centrifuge tube and the tube is spun at 3000*g* for 10 min. This results in all the RBCs settling at the bottom and the plasma sitting on the Histopaque, with the lymphocytes at the interface. Carefully remove the plasma and collect the PBLs; transfer the PBLs to another tube, wash them in physiological saline, and store the pellet at –90°C.

2. Materials

2.1. Isolation of DNA or RNA

1. Trizol LS Reagent (Life Technologies Inc., Gaithersburg, MD) for isolation of nucleic acid from liquid samples.
2. Trizol Reagent (Life Technologies) for isolation of nucleic acid from cells and tissues.
3. Phenol:chloroform: isoamyl alcohol (25:24:1, v/v, Life Technologies).
4. Molecular biology grade (DNase- and RNase-free grade) isopropanol (Sigma).
5. Ethanol.
6. Molecular biology grade sterile distilled water (conductivity 18Ω; Sigma).
7. Cloned ribonuclease inhibitor; 10,000 U/mL (Life Technologies), or RNasin (40,000 U/mL; Promega Corp., Madison, WI).

2.2. PCR

1. Recombinant *Taq* DNA polymerase (Life Technologies), includes 10X PCR buffer (200 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl).
2. 50 m*M* $MgCl_2$.
3. Either 10 m*M* dNTP solution mix (Life Technologies, Pharmacia, Uppsala, Sweden, or Boehringer Mannheim, Indianopolis, IN), or GeneAmp PCR reagent kit (Perkin-Elmer/Cetus, Norwalk, CT) containing AmpliTaq DNA polymerase, all four nucleotide solutions (10 m*M* each), 10X reaction buffer, and control template and primers. These reagents should be stored at –20°C, preferably in a Labtop cooler (Nalgene), and should be transported for short periods in the cooler.
4. Molecular biology grade mineral oil (protease-, DNase-, and RNase-free; from Sigma). Not required if using the Thermal cycler 9600, or any other cycler in which the top of the tube is also heated.
5. Oligonucleotide primers can be synthesized in-house using standard procedures or custom synthesized and purchased from any of the companies, e.g., Midland Certified Reagent Company, Midland, TX, or Bio-synthesis, Inc., Lewisville, TX. Generally, the crude-column-purified grade is adequate for PCR and RT-PCR.

2.3. RT-PCR (from Body Fluids/Tissues)

1. Superscript II RNase H-reverse transcriptase (Life Technologies), supplied with 5X first-strand buffer (250 m*M* Tris-HCl, pH 8.3, 375 m*M* KCl, 15 m*M* $MgCl_2$), and 100 m*M* dithiothreitol (DTT).

2. All reagents listed under PCR or GeneAmp complete RNA PCR kit (Perkin-Elmer/Cetus).

2.4. In Situ RT-PCR

1. Chambers for thermal cycling (Gene Cone from Gene Tec Corporation, Durham, NC).
2. Slidetemp adaptor kit (Gene Tec Corporation).
3. All reagents required for RT-PCR.
4. 11-dUTP digoxigenin.

2.5. Detection of [^{32}P]-Labeled PCR Products on Southern Blots

1. 3MM paper from Whatman or medium thickness paper grade GB002 (Schleicher & Schuell, Keene, NH).
2. Gel blot paper, GB004 (Schleicher & Schuell).
3. 0.2 µm Nitrocellulose pore size (Schleicher & Scheull).
4. 20X SSC: Dissolve 175.3 g NaCl and 88.2 g sodium citrate in 800 mL of distilled water and adjust the pH to 7.0 with 10 *N* NaOH; adjust the total volume to 1 L.
5. 50X Denhardt's solution (dissolve 1 g Ficoll, 1 g polyvinylpyrrolidone, and 1 g bovine serum albumin (BSA) in 100 mL distilled water and filter sterilize the solution).
6. 10% (w/v) Sodium dodecyl sulfate (SDS) stock solution.
7. Yeast tRNA solution (50 mg/mL) or denatured salmon sperm DNA (50 mg/mL) (Sigma).
8. [γ^{32}P] ATP, specific activity >3,000 Ci/mmol (Amersham, Arlington Heights, IL).
9. T4 Polynucleotide kinase and 5X polynucleotide kinase buffer (Gibco-BRL, Gaithersburg, MD).

2.6. Nonradioisotopic Detection of PCR Products on Southern Blots

1. 20X SSC and 10% SDS (same as in **Subheading 2.5.**).
2. Buffer 1: 0.15 *M* NaCl (8.77 g/L), 0.1 *M* Tris base (12.1 g/L), adjust to pH 7.5 with concentrated HCl and make up to 1 L.
3. Buffer 2: 0.4 *M* NaCl (23.4 g/L), 0.1 *M* Tris base (12.1 g/L), adjust to pH 7.5 with concentrated HCl and make up to 1 L.
4. 3MM paper from Whatman or medium thickness paper grade GB002 (Schleicher & Schuell).
5. Gel blot paper, GB004 (Schleicher & Schuell).
6. Hybond-N+, positively charged nylon membrane (Amersham), or maximum strength Nytran nylon transfer membrane (Schleicher & Schuell).
7. Enhanced chemiluminescence (ECL) 3'-oligo-labeling and detection systems (Amersham), or containing **items 8–15**.
8. 50 µL Flourescein-11-dUTP.
9. 100 µL Terminal transferase, 2 U/mL in a buffer solution, pH 7.0.
10. 100 µL Cacodylate buffer, 10X concentrated buffer containing sodium cacaody-late, pH 7.2.
11. Sterile deionized distiller water, RNase- and DNase-free (Sigma).
12. Blocking agent (for use in hybridization buffer and membrane blocking solution).
13. Antifluorescein horseradish peroxidase (HRP) conjugate.
14. Hybridization buffer component.
15. Detection reagents 1 and 2 (to be mixed 1:1 prior to use) provided in the detection system (**item 7**).

3. Methods

3.1. Isolation of Nucleic Acids

3.1.1. Isolation of RNA from Body Fluids (e.g. Plasma/Serum)

1. Add 750 µL Trizol LS reagent to 250 µL plasma, serum, or filtered and concentrated extract from stools/feces in a sterile 1.5-mL microfuge tube and mix at room temperature for 5 min.
2. To this mixture add 200 µL phenol:chloroform: isoamyl alcohol (25:24:1, v/v) is added and mixed.
3. Centrifuge at 12,000*g* for 15 min at 4°C.
4. Remove the colorless aqueous phase at the top and place in a new tube with 500 µL of isopropanol.
5. Centrifuge at 4°C for 10 min.
6. Gently remove the isopropanol and immerse the pellet in 1 mL 75% ethanol; centrifuge at 12,000*g* for 2 min.
7. Gently remove the supernatant and replace with 500 µL 100% ethanol and centrifuge.
8. Discard the supernatant and air dry the pellet by placing the inverted tube at an angle on a

thin pencil or a 1-mL pipet. The drying procedure can also be performed in a Speedvac (Savant Instruments, Hicksville, NY) but if not carefully done can result in the loss of the pellet or its contamination with nucleases.

9. Suspend the dry pellet in 6 µL sterile RNase- and DNase-free distilled water (18 MΩ) containing RNasin at a concentration of 1 U/10 µL.

3.1.2. Isolation of RNA Using the Qiagen Kit

This procedure eliminates the use of phenol and chloroform but requires the purchase of kits from Qiagen.

1. Mix 100 µL of plasma or serum with a buffer containing guanidium isothiocyanate.
2. Adjust the lysate to optimal binding conditions, and apply it to the spin column provided with the kit by two quick spins in a benchtop microcentrifuge. The RNA binds to the special silica-gel-based membrane and contaminants that remain separated from the adsorbed RNA by two short spin washes.
3. Elute the purified RNA in water, precipitated with ethanol, and dissolve in distilled water containing RNasin.

3.1.3. Isolation of DNA from Body Fluids

1. Add to 750 µL of Trizol LS reagent to 250 µL plasma in a sterile 1.5-mL microfuge tube and mix at room temperature for 5 min. To this mixture add 200 µL phenol: chloroform:isoamyl alcohol mixture is added and cap the tube securely.
2. Vigorously shake the tube by inverting it several times for 15 s; incubate at room temperature for 5–10 min.
3. Place the tube in a microfuge and centrifuge at 12,000*g* for 15 min at 4°C. The mixture will separate into a colorless upper phase, which contains the RNA; the interphase, which contains the protein and some DNA; and a red-colored lower phase, which contains the DNA. Carefully remove the aqueous phase overlaying the interphase, and add 0.3 mL 100% ethanol to the interphase and the lower phase; and mix by inversion.
4. Leave the samples at room temperature for 2–3 min; then centrifuge at 2000*g* for 5 min at 4°C. Remove the phenol-ethanol supernatant and discard it.
5. Wash the DNA pellet twice with 1 mL of a solution containing 0.1 *M* sodium citrate in 10% ethanol, leaving the washing solution for 30 min at room temperature (with periodic mixing); centrifuge at 2000*g* for 5 min at 4°C.
6. After the two washes, suspend the pellet in 1.5 mL 75% ethanol and store for 10–20 min at room temperature (with occasional mixing); centrifuge at 2000*g* for 5 min at 4°C.
7. Gently remove the supernatant and dry the pellet for a brief period by inverting the tube at a very slight angle; dissolve the pellet in about 100 µL of 8 m*M* NaOH by gently pipetting the solution up and down.
8. Add an adequate amount of 8 m*M* NaOH (typically, 0.3–0.6 mL per 50–70 mg of tissue or 1×10^7 cells) to obtain a DNA concentration of 0.2–0.3 µg/mL. If at this stage the preparation contains insoluble gel-like material, centrifuge at 12,000*g* for 10 min and transfer the supernatant to a new tube.

3.1.4. Isolation of RNA from Liver Tissue

1. Homogenize 100 mg of liver tissue, using a microtissue homogenizer (Fisher Scientific Co., Pittsburgh, PA) with a Teflon pestle, in 750 µL of a lysis-buffer consisting of 20 m*M* Tris-HCl (pH 8.0), 20 m*M* EDTA (pH 8.0), 2% SDS, 1 mg/mL proteinase K (Life Technologies or Boehringer Mannheim).
2. Transfer the homogenate to a microfuge tube and incubate at 60°C overnight.
3. Centrifuge at and treat about 250 µL of the resulting supernatant as described under isolation of RNA from body fluids.

3.1.5. Isolation of DNA from Liver Tissue

1. For every 50–100 mg of fresh-frozen liver tissue, add 0.75 mL of Trizol reagent and make a suspension with either a glass-Teflon or power homogenizer (Polytron or Tekmar's tissuemizer). If homogenizers are not available process tissue as described for RNA from liver tissue and then proceed as described under isolation of DNA from body fluids.
2. To extract nucleic acids (DNA or RNA) from formalin-fixed, paraffin-embedded (FFPE) liver tissue, trim the excess paraffin with a

single-edged razor blade and extract the remaining paraffin twice with 1 mL xylene for 5 min at 55°C. Wash the tissue twice with 100% ethanol and once with 50% ethanol to remove xylene; as described for fresh frozen tissue.

3.2. RT-PCR from Isolated RNA (Optimized for HCV-RNA)

1. Prepare a master mix sufficient for several different RNA samples *(n)* for the reverse transcription step as follows:
   a. $(n + 1) \times 3.5$ mL sterile deionized distiller water.
   b. $(n + 1) \times 2.0$ mL 5X buffer (250 m*M* Tris-HCl, pH 8.3, 375 m*M* KCl, 15 m*M* $MgCl_2$ (Gibco-BRL).
   c. $(n + 1) \times 1.0$ mL 0.1 M DTT (Gibco-BRL).
   d. $(n + 1) \times 1.0$ mL dNTP mix (10 m*M* each dATP, dTTP, dCTP, and dGTP, Gibco-BRL).
   e. $(n + 1) \times 0.25$ mL RNasin (10,000 U/mL [Gibco-BRL]).
   f. $(n + 1) \times 0.2$ mL (40 U) of Superscript II (Gibco-BRL).
   g. $(n + 1) \times 0.1$ mL of the antisense oligonucleotide primer (5–6 mg/mL).

   It is assumed here that $n = 10$ or more. If the number of samples is less than 10, then dilute the primers, superscript, and the RNasin 1/10 prior to adding to the master mix and adjust the water volume accordingly.
2. Gently mix the master mix and add 8 µL directly to a PCR tube containing 2 µL RNA. Incubate the tube at 43°C for 1 h, either in a water bath or in the thermal cycler. After 1 h quickly centrifuge the tube and place it in ice.
3. To this tube, add 40 µL of a PCR master mix prepared as follows:
   a. $(n + 1) \times 34$ µL of sterile, deionized distilled water.
   b. $(n + 1) \times 4$ µL 10X PCR Buffer (200 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl [Gibco-BRL]).
   c. $(n + 1) \times 2$ µL 50 m*M* $MgCl_2$.
   d. $(n + 1) \times 0.1$ µL of the sense oligonucleotide primer (5–6 mg/mL).
   e. $(n + 1) \times 0.25$ µL *Taq DNA polymerase* (5 U/µL [Gibco-BRL]).

   Mix the contents and overlay with 50 µL mineral oil.
4. Place the tubes in a thermal cycler and perform 35 cycles of PCR amplification as follows:
   a. Denature at 94°C for 1 min.
   b. Anneal at 50°C for 1 min.
   c. Extend at 72°C for 1 min.

   Perform three autoextentions for 30 s and maintain the reaction at 4°C after completion of the cycles.
5. Analyze the amplification products by mixing 16 µL PCR mix (carefully removed from under the mineral oil) and 4 µL loading buffer (0.05% w/v, bromophenol blue, 40% sucrose, 0.1 *M* EDTA, pH 8.0, and 0.5% SDS [Sigma]); load the mixture on a 2% agarose gel with *Hae*III digested phiX DNA as marker. Soak the gel in two vol of 0.5 µg/mL ethidium bromide. Place the gel on a UV light box and photograph. The amplification product should be a sharp band of the expected size (*see* **Note 5**).

3.2.1. PCR from HBV-DNA

1. Prepare the PCR master mix as follows:
   a. $(n + 1) \times 32$ µL sterile, deionized distilled water.
   b. $(n + 1) \times 5$ µL 10X PCR Buffer (200 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl [Gibco-BRL]).
   c. $(n + 1) \times 1.5$ µL 50 m*M* $MgCl_2$.
   d. $(n + 1) \times 5$ µL of 1 m*M* each primer pair.
   e. $(n + 1) \times 1$ µL of 10 m*M* dNTP mix (10 m*M* each of dATP, dTTP, dCTP, and dGTP [Gibco-BRL]) to give a final concentration of 200 m*M* each of the four dNTPs.
   f. $(n + 1) \times 0.5$ µL *Taq* DNA polymerase (5 U/µL [GibcoBRL]). Add 45 µL PCR master mix to the sample DNA in a PCR tube in 5 µL of water. Overlay the final reaction mix with 50 µL mineral oil.
3. Place the tubes in a thermal cycler and perform 35 cycles of PCR amplifications as described in **Subheading 3.2.** with one exception: the annealing is done at 60°C for 1 min. At the end of 35 cycles, place the tubes at 4°C and analyze the product as described for RT-PCR.

3.3. Confirmation of PCR Signal

3.3.1. Nested PCR

Nested PCR is the second round of PCR, using the amplification product of the first round as the template and oligonucleotides from internal or inner regions as primers.

1. Transfer a fraction of the first round of PCR product (generally 1 μL) to the second round 50-μL reaction mix prepared so that the final concentration is 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 U recombinant *Taq* DNA polymerase, 200 m*M* of each dNTP, and about 125 ng of each inner primer. Overlay with mineral oil if using the earlier versions of the thermal cycler.
2. Perform 25 cycles of PCR amplification as in **Subheading 3.2.**, followed by a 3-s auto-extention at 72°C.
3. Mix 16 μL of the PCR product with 4 μL of the gel-loading buffer (.25% w/v bromophenol blue, 0.25% w/v xylene cyanole FF, 40% w/v sucrose in water), and analyze by electrophoresis on a 2% agarose gel. Visualize bands by ethidium bromide staining and photograph. Alternatively gel loading solution can be purchased from Sigma (St. Louis, MO, cat. no. G-7654).

3.3.2. Southern Blotting

1. Transfer the gel to a glass baking dish and denature the DNA by soaking the gel in several vol of 1.5 *M* NaCl and 0.5 *M* NaOH for 1 h at room temperature with constant shaking. (The actual transfer could be performed in the same gel box that was used for separating the DNA bands on the gel.)
2. Neutralize the gel by soaking in several vol of a solution of 1 *M* Tris-HCl, pH 8.0, and 1.5 *M* NaCl for 1 h at room temperature with constant shaking.
3. Fill the cathode and the anode sides of the gel box to about half with 10X SSC solution.
4. Cut a 3MM paper with the width equal to that of the gel and about 2 in longer on either side and place on the tray. Fold the long sides equally on both sides so that both ends remain immersed. Wet the entire filter with the 6X SSC and remove all the bubbles between the tray and the filter. Place the gel in an inverted position on the filter paper with the open end of the wells facing downwards and the edges of the gel aligning with those of the tray. Cut the nitrocellulose paper to the size of the gel, float on 2X SSC in a glass dish for a few seconds and then immerse for 2 min. Place it on the inverted gel and cover with two 3MM papers immersed in 2X SSC. Remove air bubbles.
5. Place a stack of the blotting paper on the two 3MM filter papers, cover the whole setup with Saran wrap or equivalent, and weigh it with a heavy item so that the weight is distributed evenly over the blotting paper. Allow the transfer of DNA to proceed for 12–24 h.
6. Remove the wet filters and the 3MM paper above the gel. Mark the positions of the gel slots on the nitrocellulose with a pencil. Soak the nitrocellulose in 6X SSC at room temperature for 5 min.
7. Allow the nitrocellulose to dry at room temperature by laying it over a 3MM filter paper. Place the dried nitrocellulose between sheets of 3MM paper. Bake the nitrocellulose for 2 h at 80°C under vacuum in a vacuum oven. If the nitrocellulose is not to be immediately processed, store at room temperature under vacuum between the sheets of the 3MM paper.
8. Immerse the baked nitrocellulose in 6X SSC solution for 2 min.
9. Slide the baked filter into a glass tube, add about 25 mL of the prehybridization solution (5 mL distilled water, 15 mL 10X SSC, 2.5 mL 50X Denhardt's solution, 2.5 mL 10% SDS, and 50 μL tRNA at 50 mg/mL). Close the tube tightly and incubate in a rotisserie-type hybridization oven at 68°C for 2–4 h. Eliminate any bubble between the nitrocellulose and the side of the tube. Add 100 μL [^{32}P]-radiolabeled probe and 0.5*M* EDTA to the prehybridization solution and let the tube incubate at 40°C for 18–24 h. The labeled probe is prepared as follows:
   Mix 10 μL 5X buffer, 30 μL [γ^{32}P] ATP, 10 μL oligonucleotide probe (0.1 mg/mL), and 1 μL

T4 polynucleotide kinase in a 0.5-mL microtube, and incubate at 37°C for 30 min. At the end of 30 min, transfer the contents to an ultrafilter with a cutoff mol wt of 1000 containing 1 mL distilled water. Centrifuge the ultrafilter for 1 h at 5000*g*. Resuspend the concentrate in 1 mL water and concentrate as before by centrifugation. The concentrated solution-free of the unincorporated label is the probe.

10. After the hybridization is complete, discard the liquid from the glass tube into the radioactive liquid waste container or in a sink designated for radioactive waste disposal. Wash the nitrocellulose in a glass dish containing 2X SSC and 0.5% SDS at room temperature for 5 min.
11. Discard the radioactive wash solution as before and replace with a solution of 2X SSC and 0.1% SDS; wash at room temperature with shaking for 15 min. Replace the solution with 0.1X SSC and 0.5% SDS and incubate in the oven at 40°C for 2 h. Discard the final wash and wrap the wet filter in Saran wrap. Expose the filter to an X-ray film for sufficient time to see a signal at –80°C and develop in a film developer.

3.3. Nonisotopic Detection of Signal

Follow **steps 1–3** in **Subheading 3.3.2.**, and continue with **step 4**.

4. Cut a 3MM paper with the width equal to that of the gel and about 2 in longer on either side and place on the tray. Fold the long sides equally on both sides so that both ends remain immersed. Wet the entire filter with the 6X SSC and remove all the bubbles between the tray and the filter. Place the gel in an inverted position on the filter paper with the open end of the wells facing downwards and the edges of the gel aligning with those of the tray. Cut the nylon membrane to the size of the gel; float on 2X SSC in a glass dish for a few seconds and then immerse for a couple of min. Place it on the inverted gel and cover with two 3MM papers immersed in 2X SSC. Remove air bubbles.
5. Place a stack of the blotting paper on the two 3MM filter papers, cover the whole setup with Saran wrap or equivalent, and weigh it with a heavy item so that the weight is distributed evenly over the blotting paper. Allow the transfer of DNA to proceed for 12–24 h.
6. Remove the wet filters and the 3MM paper above the gel. Mark the positions of the gel slots on the nylon membrane with a pencil. Soak the nylon membrane in 6X SSC at room temperature for 5 min.
7. Allow the nylon membrane to dry at room temperature by laying it over a 3MM filter paper. Place the dried nylon membrane between sheets of 3MM paper. Bake the nylon membrane for 2 h at 80°C under vacuum in a vacuum oven.
8. Perform the 3'-end labeling of the oligonucleotide as follows:
   Mix 100 pmol oligonucleotide, 10 µL fluorescein-dUTP, 1 6 µL cacodylate, and 16 µL terminal transferase in a 0.5-mL tube and bring the total vol up to 160 µL. Incubate the tube for 60 min at 37°C. The labeled probe may be stored at –20°C in a nonfrost-free freezer.
9. Float the filter in 6X SSC for 2 min and prehybridize the blot by placing it in a glass tube, adding to the tube about 50 mL of prehybridization solution (5X SSC, 0.1% (w/v) hybridization buffer component, 0.02% (w/v SDS, and 0.5% blocking agent). Alternatively, the prehybridization solution from **Subheading 3.3.2.** may be used. Prehybridize in a rotisserie oven at 42°C at least 30 min, but this may go overnight.
10. Hybridize by adding 10 ng/mL probe to the prehybridization solution in the tube; place in the rotisserie oven at 42°C for a minimum of 1–2 h.
11. At the end of the hybridization period remove the solution and replace it with 5X SSC and 0.1% SDS. Wash the blot twice at room temperature for 5 min, agitating on a belly dancer shaker. Perform a stringency wash at 42°C twice for 15 min each time with 0.1–1X SSC and 0.1% SDS with agitation. Rinse the blot in buffer 1 for 1 min and then incubate in the block buffer for 30 min. Rinse in buffer 1 again, and place in a solution containing the antifluorescein antibody at a 1:1000 dilution in 0.5% BSA in buffer 2 and incubate for 30 min. Wash the blot four times in buffer 2 for 5 min each time. Detection of the signal is performed by incubating the blot in the solution (0.125 mL/

cm_2) containing equal vol of the two detection reagents for 1 min. Drain the blot against the sides of the tray or tube and cover with Saran wrap. Immediately expose to X-ray film. Generally the amount of time required to see a signal is <1 h.

3.4. Quantitation of Nucleic Acids

3.4.1. Branched DNA/RNA Assay

The following brief protocol was prepared from information provided by Chiron Corp. (Emeryville, CA). It requires the use of Quantiplex HBV-DNA assay (bDNA) kit for determining the direct quantitation of HBV load in serum or HCV-RNA (bDNA) kit for determining the direct quantitation of HCV load in serum. Chiron also requires any new user to take a course on the assay's use and a detailed protocol is provided with the kit. This assay is highly specific, and reliable, and requires only about 10 µL of the serum specimen.

1. To perform the HBV-DNA assay, mix 10 µL specimen with 10 µL extraction buffer for 30 min at 63°C. To this mixture add 10 µL denaturation buffer and HBV probes and mix once more at 63°C for 30 min.
2. Add 10 µL of neutralization buffer to the mixture and place in wells.
3. Incubate at 63°C for 16–18 h and wash the plate.
4. Add 50 µL amplifier (bDNA) to the washed wells and incubate at 53°C for 30 min, then wash.
5. Add 50 µL labeled probe at 53°C for 15 min and wash.
6. Add 50 µL chemiluminescent substrate to the mixture and incubate at 37°C for 25 min. Measure the light emission and determine the HBV-DNA equivalents/mL in the specimens using a standard curve.

3.4.2. Colorimetric Assay

The following protocol was prepared using information provided by Roche Diagnostic System, Inc. (Branchburg, NJ) and it requires the use of Amplicor HCV Monitor kit. Roche also requires any new user to take a course on use of the assay. It is currently suggested to be used only as a research tool for monitoring treatment. The principle is as follows: RNA is prepared using standard procedures described above, but is suspended in a buffer containing manganese, necessary in the PCR procedure. The RNA is then placed in a combined reverse transcription-PCR reaction, referred to as the tRT-PCR reaction, that uses a single set of primers, a single enzyme (DNA polymerase *Thermus thermophilus* rTth1 instead of reverse transcriptase and *Taq* polymerase), and one set of optimized buffer conditions. A 244-p amplification product termed the amplicon is obtained. The amplicon is denatured and transferred to a special microwell detection plate coated with a unique probe sequence specific for capturing the amplicon but not overlapping with the primers. Horseradish peroxidase conjugated to avidin binds to the amplicon when added, and the color is produced when the substrate tetra methylbenzidine (TMH) is added. The color produced is measured at 450 nm and is proportional to the amount of DNA.

3.5. Serological Assay for Detection of Antibody

Serological assays vary mainly with respect to the antigen used; once the antigen is bound to a 96-well plate (ELISA) or beads or nitrocellulose (RIBA), the subsequent procedure is more or less identical. Two different substrate solutions can be used depending on whether the secondary antibody is coupled to horseradish peroxidase or phosphatase. If the secondary antibody is conjugated to peroxidase, the substrate solution of H_2O_2 as the substrate and 1,2 phenylenediamine dehydrochloride as the color-forming compound is used, and the yellow color product is measured at 450/490 nm.

3.5.1. ELISA for Hepatitis A Virus

Prior to discussing a specific ELISA/EIA, the various commercially available plates coated with different antigens for the antibody to the different hepatitis viruses is discussed.

Two enzyme immunoassay kits are available from the Abbott Laboratories Diagnostic Division (Chicago, IL) with a detailed protocol. A sandwich assay specific for IgM antibody (indicative

of the acute phase) to HAV allows its qualitative detection in human serum or plasma as follows. Beads coated with goat antihuman antibody specific for human IgM (μ-chain) are incubated with either the controls or diluted specimens at 40°C ± 1°C for about 1 h. The IgM from the sample binds to the antibody on the beads. All unbound material is washed. HAV antigen is mixed with the beads and it binds to any anti-HAV IgM present on the beads. Unbound HAV Ag is washed away and Human anti-HAV conjugated with horseradish peroxidase is mixed and incubated with the beads; it will bind to the HAV Ag present on the beads. Unbound conjugate is washed and the beads are incubated with the substrate solution. The color development procedure is described in **Subheading 3.5.3.** The absorbance is directly proportional to the amount of anti-HAV IgM present in the specimen.

In a second assay (competition), the qualitative and semi-quantitative detection of total antibody to HAV is achieved. This assay is mainly performed to determine previous exposure to HAV for assessing immune status or for epidemiologic studies. Beads coated with formaldehyde inactivated HAV are mixed with the controls or the specimens and anti-HAV antibody conjugated with horseradish peroxidase. The anti-HAV antibody in the sample competes with the limited number of HAV binding sites present on the beads. All unbound material is washed away and the beads are incubated with the substrate solution as described in **Subheading 3.5.3** in detail. The intensity of the color that develops is inversely proportional to the amount of anti-HAV in the sample.

3.5.2. ELISA for Hepatitis B Virus

Abbott Diagnostics markets ELISA kits accompanied by detailed protocols (essentially the same as those for HAV described in **Section 3.5.1.** or HCV described in **Subheading 3.5.3.** for the following markers: HBsAg, HBeAg, anti-HBc (total antibody to hepatitis B core antigen), anti-HBc IgM (IgM antibody to hepatitis B core antigen), anti-HBs (antibody to hepatitis B surface antigen). In addition, Organon Technikon (Chicago, IL) also has an ELISA kit for anti-HBs.

3.5.3. HCV-SP ELISA for Detection of HCV-Specific Antibodies

A polypeptide HCV-SP is synthesized. Each well of 96-well ELISA plates is coated with 100 ng HCV-SP by adding 100 μL of a 1 μg/μL stock solution of the peptide followed by washing with 200 μL of PBS:0.1% Tween. Serum from patients or healthy donors is pretreated with 0.5% NP40 to a final concentration of 0.1% NP40 and diluted 1:5 in PBS:Tween and 100 μL diluted serum sample is placed in a well and the plate is covered and incubated on a shaker for at least 4 h at room temperature or 1 h at 37°C. At the end of the incubation, the plate is washed at least 5 times with 200 μL of PBS:Tween. A mixture of rabbit antihuman IgG and IgM conjugated to horseradish peroxidase diluted 1:500 is added to the wells and the plate is incubated at room temperature for 1–4 h. At the end of the incubation the antibody solution is discarded and the plate washed at least five times. Absorbance of the orange color formed in addition to 200 μL of soluble peroxidase substrate solution—prepared fresh by dissolving two tablets from Sigma referred to as Sigmafast OPD tablets (one is *o*-phenylenediamine dehydrochloride [OPD] and the other is urea H_2O_2) to make 20 μL of buffered substrate—is read on a microwell plate reader at 492 nm in about 15 min.

3.5.4. Antibody to Hepatitis D Virus

This can be measured by an anti-hepatitis δ ELISA kit from Abbott. The protocol is essentially similar to the ones described above.

4. Notes

1. Handling of RNA requires extreme care. Always wear gloves when holding samples and reagents. Avoid freezing and thawing of samples and reagents. Never let individual reagents reach room temperature. Ensure that the reagents are used by only one or two persons. If there are multiple users, aliquot the reagents. The tubes used for the reactions and any pipet tips should

be autoclaved prior to each use. If changes are to be made in the source of a reagent, make the change one at a time and perform a run with the old and new reagent side by side in a given reaction prior to switching to the new reagent.

2. PCR and RT-PCR are susceptible to contamination from aerosol resulting in false-positive reactions and it is therefore essential that these procedures be performed in a separate room or an isolated corner designated solely for this purpose. After each use the pipetman should be placed in a 50 mL conical tube containing about 5 mL of 1% SDS and 1 m*M* EDTA solution. Each time the pipetman is removed from the solution, shake off all the dripping liquid otherwise it will enter the reaction mixture.
3. Since there are multiple additions for the PCR and RT-PCR reactions it is very important to make a note (tick mark) each time a reagent is added, so that none of the reagents are missed or added more than once.
4. The enzymes such as Superscript and *Taq* are generally in glycerol solutions, making their solutions very viscous. Always make sure that these enzymes are added last and the reaction is mixed by pipetting up and down with the microtip.
5. Run several internal and external controls. An internal control would be previously tested known positive and negative RNA plasma samples processed at the same time as the batch of samples and in exactly the same fashion. Thus, a problem in the isolation will be detected. Also, run a known positive and negative RNA, previously isolated and found to give the required outcome. These will also serve as internal controls of the RT-PCR procedure. In addition to these internal controls, run an external control along with the amplified product on the gel. This control would be a known RT-PCR positive product of the correct size and a negative control showing no product from a previous amplification of a sample negative for HCV. This will serve both as a size marker as well as an indication of whether the set of reactions from the current lot are comparable to the previous lot. In addition to the samples and internal controls, always run a sample including all the reagents, except the RNA or DNA. This should not give rise to any product, but if it does it will mean there is some contamination of the reagents or oligonucleotides with the nucleic acid being tested. In this case, it is imperative to determine which reagent is contaminated by the process of elimination and discard that reagent. Alternatively, all reagents can be replaced with new ones.

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