Diagnostic Methods of HBV infection

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Hepatitis B-laboratory diagnosis

Detection of HBV infection involves detecting the presence of:
– Viral genetic material
– Viral proteins (antigens)
– Antibody response to viral antigens
Diagnosis of acute hepatitis B

Diagnosis of HBV infection has generally relied on interpretation of hepatitis B specific serology and biochemical markers of liver damage.

There is currently no role for molecular testing in the diagnosis of acute hepatitis B other than in the detection of asymptomatic patients during pretransfusion screening of blood products.
Serological Tests for HBV

- Antibody assays
  - anti-HBs
  - anti-HBe
  - anti-HBc
    - IgM
    - Total
- Antigen assays
  - HBsAg
  - HBeAg

Confirmatory Assays:
HBsAg neutralisation
Interpretation of common HBV serology patterns

<table>
<thead>
<tr>
<th>HBsAg</th>
<th>HBeAg</th>
<th>IgM Anti-HBc</th>
<th>IgG Anti-HBc</th>
<th>Anti-HBe</th>
<th>Anti-HBs</th>
<th>HBV-DNA</th>
<th>Interpretation</th>
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<tbody>
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<td>-</td>
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<td>Susceptible to HBV Early in HBV incubation period</td>
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<td>+</td>
<td>Early in acute HBV Possible HBV variant infection</td>
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<td>Early in acute HBV</td>
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<td>Early in acute HBV</td>
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<td>+</td>
<td>Chronic HBV (high infectivity)</td>
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<td>Window period acute HBV</td>
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<td>+</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>low infectivity Non-replication</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Core mutation</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Recovery</td>
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<td>-</td>
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<td>+</td>
<td>Immunization response Convalescence from HBV with waning anti-HBc and anti-HBe Passive immunoprophylaxis by Ig</td>
</tr>
</tbody>
</table>

**Interpretation of common HBV serology patterns**

- **HBsAg**: Hepatitis B surface antigen
- **HBeAg**: Hepatitis B e antigen
- **IgM**: Immunoglobulin M
- **IgG**: Immunoglobulin G
- **Anti-HBc**: Antibody to hepatitis B core antigen
- **Anti-HBe**: Antibody to hepatitis B e antigen
- **Anti-HBs**: Antibody to hepatitis B surface antigen
- **HBV-DNA**: Hepatitis B virus DNA

**Interpretations**:
- **Susceptible to HBV**: Early in HBV incubation period
- **Early in acute HBV**: Possible HBV variant infection
- **Chronic HBV (high infectivity)**: Window period acute HBV
- **Non-replication**: Low infectivity
- **Core mutation**: Recovery
- **Immunization response**: Convalescence from HBV with waning anti-HBc and anti-HBe Passive immunoprophylaxis by Ig
Discordant or unusual hepatitis B serologic profile requiring further evaluation

Positive for HBs Ag, anti-HBs, anti-HBc

- HBs Ag is from one HBV-subtype and anti-HBs is directed to another HBV subtype
- Simultaneous or sequential infection with different subtypes of HBV
- During resolution of acute HBV infection
- Abnormal, nonspecific immune response to a single subtype

HbeAg positive/ HBsAg negative

- anti-HBs Positive only in a non immunized person

Positive for HBe Ag and anti-HBe

- anti-HBc Positive only
Isolated anti-HBc profile

Late acute resolving infection when HBsAg undetectable and low level HBV-DNA

Chronic carrier state where HBsAg level are blow the detection limit of current assay

Mutation in virus with low –level replication or altered epitopes

False positive
Suggested algorithm for evaluation of patients with isolated hepatitis B core antibody (HBcAb)

1. Isolated HBcAb
   - Repeat test for HBcAb, HBsAg, HBsAb
   - Test for HBeAg, HBeAb, HCV Ab
   - Vaccinate with single dose of HBV vaccine and measure HBsAb one month later

2. HBsAb ≥ 10 IU/mL (anamnestic response)
   - IMMUNE
   - No further vaccination

3. HBsAb < 10 IU/mL
   - Complete 3 doses of vaccination and measure HBsAb one month after final dose

4. Undetectable HBsAb
   - POTENTIALLY INFECTED WITH HBV
   - Consider HBV DNA testing, screening and vaccination of contacts

5. Positive HBsAb
   - IMMUNE
Discordant or unusual hepatitis B serologic profile requiring further evaluation

HBsAg positive and anti-HBc negative

Mutation core gene region (mutation in B cell / T cells epitopes)

Immune tolerance during in utero infection HBV

Early acute infection before the development of anti-Hbc ab
Four types of molecular assays are available for the diagnosis and management of HBV infection:

- Qualitative & quantitative (viral load) tests
- Genotyping assays
- Drug resistance mutation tests
- Core promoter/pre core core mutation assays
Problems with PCR

1) Specimen processing and template degradation
   - Proper specimen transport
   - Processing and Storage

2) Inhibitors
   PCR amplification depends upon the enzymatic activity of a DNA polymerase, PCR can be inhibited by any substance (heparin, hemoglobin, lactoferrin) that negatively affects synthesis by DNA polymerase. Spiking the specimens being analyzed with an internal positive control can assess whether inhibitors are present.
3) Primer defects
   - cross-annealing
   - primer-dimer formation
   - Hairpin loops

4) Contamination between specimens causes false-positive results, and even more likely source of contamination is from amplicons generated in previous amplification reactions.
To minimize the potential contamination,

1) PCR laboratory are routinely set-up in only one direction

2) Special plugged tips that minimized both pipette contamination and sample aerosolization are used

3) Adding dUTP and Uracil-DNA glycosylase (UNG) to PCR reactions
viral load testing is useful:

• Before therapy
  baseline to predict the response to antivirals and the emergence of antiviral resistance, particularly to lamivudine

• During therapy

  viral load can be used as a primary end point to assess the response to nucleoside antiviral therapy

• In HBeAg- CHB, HBV DNA is the only virologic marker that can guide the decision to end treatment
Importantly, molecular assays have begun to play increasingly significant roles in chronic hepatitis B (CHB).

<table>
<thead>
<tr>
<th>Phase</th>
<th>ALT</th>
<th>HBsAg</th>
<th>HBeAg</th>
<th>HBeAg antibody</th>
<th>HBV DNA (IU/ml) (^a)</th>
<th>Liver histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune tolerance</td>
<td>Usually normal</td>
<td>Present</td>
<td>Present</td>
<td>Absent</td>
<td>(\geq 20,000)</td>
<td>Usually normal; can have mild inflammation</td>
</tr>
<tr>
<td>Immune clearance</td>
<td>Elevated; can be episodic</td>
<td>Present</td>
<td>Present</td>
<td>Absent</td>
<td>(\geq 20,000)</td>
<td>Active inflammation</td>
</tr>
<tr>
<td>Inactive HBsAg carrier</td>
<td>Usually normal; can have flares</td>
<td>Present</td>
<td>Absent</td>
<td>Present</td>
<td>(&lt;20,000^*)</td>
<td>Degree of abnormality dependent on disease severity during clearance phase (mild inflammation to inactive cirrhosis)</td>
</tr>
<tr>
<td>HBeAg^- CHB</td>
<td>Periodic flares</td>
<td>Present</td>
<td>Absent</td>
<td>Present</td>
<td>(&gt;20,000) or &lt;20,000)</td>
<td>Active inflammation</td>
</tr>
<tr>
<td>Occult hepatitis B</td>
<td>Can be elevated</td>
<td>Absent</td>
<td>Absent</td>
<td>Present in recovered HBV infection</td>
<td>(&lt;20,000^\dagger)</td>
<td>Ranges from normal to cirrhosis and HCC</td>
</tr>
</tbody>
</table>

\(^a\) Symbols: *, can be undetectable by PCR; \(^\dagger\), usually (often detectable only with highly sensitive molecular tests).
The different types of nucleic acid molecular techniques

• Direct probe testing
First-generation assays for HBV DNA quantification in peripheral blood were based on solution Hybridization technology that they are better for identification than for detection because it is not as sensitive as amplification methods.

• Amplification methods – used to improve the sensitivity of the nucleic acid testing technique that led to the development of second-generation assays with enhanced sensitivity (as low as 200 copies/ml)

Target amplification
Probe amplification
Signal amplification

• Combinations of the above
The latest generation HBV quantification assays utilize real-time PCR and have improved analytical performance characteristics, including low limits of detection, broad linear ranges, excellent precision, better
### Commercially available assays and reagents for HBV DNA quantification

<table>
<thead>
<tr>
<th>Test or reagent (manufacturer)</th>
<th>Method</th>
<th>sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>VERSANT HBV DNA 3.0 (Bayer Diagnostics) bDNA</td>
<td>semi-automated bDNA signal amplification</td>
<td>2000 (Europe, CE)</td>
</tr>
<tr>
<td>COBAS Amplicor HBV</td>
<td>Semi automated quantitative PCR</td>
<td>200 (Europe, CE)</td>
</tr>
<tr>
<td>Monitor (Roche Diagnostics)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COBAS TaqMan 48 HBV (Roche Diagnostics)</td>
<td>Real-time PCR</td>
<td>35 &lt; 50 (Europe, CE)</td>
</tr>
<tr>
<td>Artus HBV PCR (QIAGEN Diagnostics)</td>
<td>Real-time PCR</td>
<td>2*10^1 &lt; 50 (Europe, CE)</td>
</tr>
<tr>
<td>Real-Time HBV PCR (Abbott Molecular)</td>
<td>Real-time PCR</td>
<td>10 (Europe, CE)</td>
</tr>
</tbody>
</table>
Another real-time assay, the Artus RealArt HBV PCR kit (artus-biotech), contains ready-for-use reagents for the quantification of HBV DNA specifically designed for different real-time platforms.

Reagents are provided for use on the ABI Prism instruments 7000/7700/7900 (Applied Bio systems) employing the Taq-Man technology.

For the Light Cycler instrument (Roche Diagnostics), the assay uses the fluorescence resonance energy transfer (FRET) technology to monitor the fluorescence intensity.

The RealArt kit for the Rotor-Gene instrument (Corbett Research) relies on molecular beacons for detection.

In general, the linear dynamic range of the assay for all of the instruments is from approximately $10^2 - 6 \times 10^8$ copies/mL ($2 \times 10^1 - 10^8$ IU/mL).
A variety of methods have been used including

* whole- or partial-genome sequencing

* Restriction fragment length polymorphism (RFLP)
* Genotype-specific PCR amplification

* PCR plus hybridization PCR has been adapted into a commercial product (INNO-LiPA; Innogenetics)
* Serology
Drug Resistance Mutations

The emergence of drug-resistant HBV is related with a 10-fold increase in viral load compared to native is confirmed in a patient with documented therapeutic response.

Based on therapy, there are three mutants of HBV:

- Vaccine / HBIG escape mutants
- Nucleoside analogue resistance
- PreCore promoter and preCore gene changes
Genotype- and vaccine-escape induced specific exchanges in the α determinant of SHBs

- P120T
- D144E
- G145R
HEPATITIS B VIRUS GENOME

Cross resistance
Drug Resistance Mutations

HBV Polymerase

Terminal Protein | Spacer | Reverse Transcriptase | RNase H

LAM

V173L
L180M
M204V/I (YMDD)

ADV
A181V
N236T
Drug resistant mutation can be documented by

• Phenotypic analysis

• Direct sequencing can identify known and potential new resistance mutations

• PCR plus hybridization PCR that the second-generation product (INNO-LiPA DR, version 2.0) has a refined, expanded lamivudine resistance panel (codons 80, 173, 180, and 204) and also detects adefovir resistance mutations (codons 181 and 236)

Documenting the mutation that confers drug resistance has not been part of routine clinical practice.
Precore promoter/ precore mutations

The most important precore promoter mutations are A to T at nucleotide 1762 (A\textsubscript{1762} T) and G to A at nucleotide 1764 (G\textsubscript{1764} A / T) that decreased HBeAg expression.

The most important precore mutation is G to A at nucleotide 1896 (G\textsubscript{1896} A) that stopped HBeAg expression.
Detection of Core Promoter/Precore Mutations in HBeAg negative CHB

The diagnosis of HBeAg negative CHB is made primarily through assessment of a combination of virological markers (HBsAg positive, HBeAg negative, detectable HBV DNA), serology (anti-HBeAg antibody positive), and evidence of hepatic injury (elevated aminotransferases and or histologic evidence
### Interpretation of common HDV tests

<table>
<thead>
<tr>
<th>HDV- RNA</th>
<th>IgM anti-HDV</th>
<th>Anti-HD Total</th>
<th>HBsAg</th>
<th>IgM anti-HBc</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Early in acute HDV infection (Coinfection)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Late in acute HDV infection (Coinfection)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>acute HDV infection (Super-infection)</td>
</tr>
</tbody>
</table>
### Hepatitis D tests

<table>
<thead>
<tr>
<th>Test</th>
<th>Description</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HDV IgG antibody</td>
<td>Indicates previous or ongoing contact with HDV</td>
<td>The first diagnostic test Should be performed in all HBsAg + patient</td>
</tr>
<tr>
<td>Anti-HDV IgM antibody</td>
<td>Indicates acute HDV infection or chronic</td>
<td>Can be used to determine disease activity in patient who was anti- HDV-Ab+</td>
</tr>
<tr>
<td>HDV RNA qualitative</td>
<td>Indicates HDV replication and acute infection</td>
<td>Gold standard HDV infection to determine HDV infection</td>
</tr>
<tr>
<td>HDV RNA quantitative</td>
<td>Determine the level of HDV RNA in the blood</td>
<td>Can be useful in the context of antiviral treatment to predict treatment response</td>
</tr>
<tr>
<td>HDV genotyping</td>
<td>Determine HDV the genotype</td>
<td>Different HDV genotype may be associated with distinct clinical courses</td>
</tr>
<tr>
<td>Test</td>
<td>Description</td>
<td>Details</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------</td>
</tr>
<tr>
<td>HBsAg quantitative</td>
<td>Determine the level of HBsAg in the blood</td>
<td>can be useful during antiviral treatment. HBsAg is related with HDV-RNA level. HBsAg clearance is associated HDV clearance.</td>
</tr>
<tr>
<td>HBeAg/HbeAb</td>
<td>Determine the presence of the HBeAg/HbeAb</td>
<td>About 15-20% of patient with HDV infection test is positive for HBeAg, which can be associated with HBV replication for treatment with HBV polymerase inhibitor (if INF-a treatment is not possible)</td>
</tr>
<tr>
<td>HBV DNA quantitative</td>
<td>Determine the level of HBV DNA in the blood</td>
<td>for treatment with HBV polymerase inhibitor depends on the amount of HBV DNA detectable in the blood</td>
</tr>
<tr>
<td>Anti –HCV antibody/HCV RNA</td>
<td>Determine the presence of Anti –HCV antibody/HCV RNA</td>
<td>Up to one third of patient in Europe with HDV infection are co infected with HCV. HCV-RNA is suppressed by coinfection</td>
</tr>
<tr>
<td>Liver biopsy</td>
<td>For staging of liver disease</td>
<td>Predict the stage of liver disease in patients with HDV infection</td>
</tr>
</tbody>
</table>
Molecular Tests for Hepatitis
**Hepatitis Virus – Molecular Tests**

- Molecular assays available as follows:
  - Commercial assays for HBV DNA and HCV RNA
  - In-house assays for HAV RNA & HDV RNA
  - No molecular assay for HEV RNA

- HCV RNA & HBV DNA, plasma or serum must be separated from cells within 6 hrs and plasma can be stored at 4°C for several days or -70°C for long-term

- No licensed tests for diagnostic purposes; all tests are for monitoring or donor screening
  - HCV RNA will be done in HIV or other immunocompromised patients if requested
Hepatitis Virus – Molecular Tests

- Lower limit of Detection (LLD) does not equal dynamic (linear) range of quantitative assays
  - Determined by PROBIT analysis to determine the value that is consistently detected 95% of the time

- Results of different assays may (HBV) or may not (HCV) be interchangeable
Nucleic Acid Amplification Tests (NAAT) for Detection of RNA/DNA

- Quantitation of RNA or DNA may be reported as copies/ml or IU/ml

- Conversion factor for copies/ml to IU/ml is not the same for different assays measuring the same target or different targets
  - HBV DNA: 5.82 copies/IU
  - HCV RNA: PCR - 2.4 copies/IU; bDNA: 5.2 copies/IU

- Coefficient of variation (COV) may range from 15 to 50%
Diagnostics in Viral Hepatitis: Summary

- Serology remains the cornerstone for diagnosis and screening
- NAAT is critical to patient management
- Of the many NAAT tests available, PCR, bDNA and TMA remain most popular
  - Sensitivity and dynamic range varies between assays
  - Standardization allows (to some degree) interchangeability of the results with different assays
- Resistance/Genotyping requires amplification first
  - Increasing role in making treatment decisions as more drugs become available for HBV