Molecular Diagnosis of Hepatitis B and Hepatitis D infections
Acute infection

Detection of HBsAg in serum is a fundamental diagnostic marker of HBV-infection

HBsAg shows a strong correlation with HBV replication only in the early phases of infection

HBV –DNA is a direct marker for infectivity but in the early phase of infection
Acute infection

HBsAg-
- with no clinical symptoms
- with clinical symptoms

HBV infection unlikely

Test for HBV DNA
- HBV infection unlikely
+ HBV infection

Consider test for HDV Ab

HDV Ab-
HDV infection unlikely
HDV Ab+
HBV/HDV infection

HCV Ab-
HCV infection unlikely

HCV Ab+
Confirm active infection with a sensitive HCV RNA test

HCV RNA+
Active HCV infection

HCV RNA-
Active HCV infection unlikely; follow up testing as clinically indicated

HAV
HEV
Acute infection

Mutations in the S gene can lead to loss of recognition by the antibodies used in commercial assays

HBV mutations can emerge primarily in response to therapy, for instance during passive antibody therapy with high titer immunoglobulins, and after HBsAg vaccination of young, HBV-exposed children

HBsAg negative patients with hepatitis of unknown etiology should be tested with a secondary assay, e.g., viral genome detection
chronic HBV infection

The correlation of HBsAg and HBV-DNA was weak or missing when analyzing the different phases of persistent HBV-infection separately.
The “immune tolerant” phase is characterised by HBeAg positivity, high levels of HBV replication (reflected by high levels of serum HBV DNA), normal or low levels of aminotransferases, mild or no liver necroinflammation and no or slow progression of fibrosis.

The “immune reactive HBeAg-positive phase” is characterised by HBeAg positivity, relatively lower level of replication compared to the immune tolerant phase (as reflected by lower serum HBV DNA levels), increased or fluctuating levels of aminotransferases, moderate or severe liver necroinflammation and more rapid progression of fibrosis compared to the previous phase.
Natural history of chronic HBV infection

The “inactive HBV carrier state” may follow seroconversion from HBeAg to anti-HBe antibody.

It is characterised by very low or undetectable serum HBV DNA levels and normal serum aminotransferases.

ALT levels should remain persistently within the normal range according to traditional cut-off values (approximately 40 IU/ml) and HBV DNA should be below 2000 IU/ml.

The combination of serologic markers, including quantification of HBsAg and HBV DNA in serum, has improved the diagnosis of inactive carriers, which usually have a low viral load (HBsAg < 1,000 IU/mL and HBV DNA < 2,000 IU/mL).
Natural history of chronic HBV infection

**HBeAg-negative CHB**” may follow seroconversion from HBeAg to anti-HBe antibodies during the immune reactive phase or may develop after years or decades of the inactive carrier state.

It is characterised by periodic reactivation with a pattern of fluctuating levels of HBV DNA and aminotransferases and active hepatitis.
Natural history of chronic HBV infection
 occult HBV infection (OBI)

In the “HBsAg-negative phase” after HBsAg loss, low-level HBV replication may persist with detectable HBV DNA in the liver

Occult HBV infection [detectable HBV DNA in the liver with low level (<200 IU/ml) or undetectable HBV DNA in blood] by sensitive PCR assays may reflect the persistence of infected hepatocytes after the clinical resolution of the disease

Immunosuppression may lead to HBV reactivation in these patients
Natural history of chronic HBV infection
Diagnosis of occult HBV infection

Isolated HBcAb may be due to:
- Remote infection (immune or chronic carrier)
- “Window” period between HBsAg and HBsAb
- Co-infection with HCV
- False positive test result – HBcAb is marker most prone to false positives

Detection of HBV DNA by sensitive PCR assays
HCV co-infected patients

In HBV-infected patients, HCV co-infection accelerates liver disease progression and increases the risk of HCC. HBV and HCV replicate in the same hepatocyte without interference.

A proportion of these patients may have fluctuating serum HBV DNA levels. HBV DNA level is often low or undetectable and HCV is responsible for the activity of chronic hepatitis in most patients.

Thus, patients should usually receive treatment for HCV; there is a potential risk of HBV reactivation during treatment or after clearance of HCV.

Therefore, HBV DNA monitoring is necessary.
Hepatitis Virus – Molecular Tests

Molecular assays available as follows: (qualitative/quantitative)

Determine need to treat chronic HBV infection

Indicator of chronic hepatitis after diagnosis of acute HBV Infection
Indicate emergence of resistant variants during antiviral therapy and viral replication in patients with mutant HBV

Routine monitoring on /off therapy to assess response to treatment
Real-time PCR for HBV DNA

Real time PCR for HBV DNA has reached an excellent level of performance with a detection limit close to the theoretical minimum of 1 DNA molecule per reaction mix and a huge dynamic range up to $10^7$ or more.

In 1991 the WHO introduced International Standard preparations and an arbitrary International Unit (IU) of HBV DNA

The number of molecules per IU depends on the assay; but typically 5 molecules correspond to one IU HBV DNA
<table>
<thead>
<tr>
<th>Assay</th>
<th>LLD (copies/ml)</th>
<th>Linearity (copies/ml)</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versant bDNA v3.0 (Siemens)</td>
<td>2 x 10^3</td>
<td>2 x 10^3 to 1 x 10^8</td>
<td>15 - 37%</td>
</tr>
<tr>
<td>Hybrid Capture II (Digene)</td>
<td>5 x 10^3</td>
<td>5 x 10^3 to 6 x 10^7</td>
<td>10 – 15%</td>
</tr>
<tr>
<td>Real-Time HBV PCR (Abbott Molecular)</td>
<td>10</td>
<td>5 x 10^5 to 1 x 10^10</td>
<td>12 – 22%</td>
</tr>
<tr>
<td>Cobas Taqman (Roche)</td>
<td>35 (Manual)</td>
<td>2 x 10^2 to 1 x 10^10</td>
<td>16 – 54%</td>
</tr>
<tr>
<td></td>
<td>70 (Automated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RealArt HBV PCR (artus/Qiagen)</td>
<td>10</td>
<td>1 to 4 x 10^8</td>
<td></td>
</tr>
</tbody>
</table>

1 IU/mL = 5.82 copies/mL
### Disease Phases in Chronic HBV Infection

<table>
<thead>
<tr>
<th>Phase</th>
<th>HBsAg</th>
<th>HBeAg</th>
<th>Anti-HBe</th>
<th>ALT</th>
<th>HBV DNA range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune Tolerant</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Normal</td>
<td>&gt;8 log IU/mL</td>
</tr>
<tr>
<td>Immune Clearance</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Normal or elevated</td>
<td>3-8 log IU/mL</td>
</tr>
<tr>
<td>Inactive Disease</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Normal</td>
<td>&lt;3 log IU/mL</td>
</tr>
<tr>
<td>HBeAg-negative Chronic</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Normal or elevated</td>
<td>3-8 log IU/mL</td>
</tr>
</tbody>
</table>
Laboratory Tests for HBV

- HBV genotyping
- HBV drug resistance testing
- BCP/PreCore Mutants

**Molecular Methods include:**

- RFLP of PCR- amplification products
- Sequencing
- Hybridization (Line Probe Assay, Trugene Assay)
Hepatitis B – Laboratory Tests

Genotyping

Genotyping of isolates for epidemiological purposes; categorizes patient isolates into 8 different HBV genotypes (A to H)

Genotype has also been associated with response to interferon-based therapy
Genotyping

- Genotype analysis may have prognostic value. Genotype C has been associated with more severe liver disease than genotype B.

- Genotype D is observed mainly in the Mediterranean basin and is associated with a higher prevalence of pre-C mutants in chronically infected individuals.
Hepatitis B – Laboratory Tests

Basal core promoter/Precore mutants

Due to mutation in at nucleotide 1896 of the precore (abolishes HBeAg production)

or at nucleotides 1762 and 1764 of the BCP regions (down-regulates HBeAg production)

No effect on viral replication. Patients with mutations in either region can return to high levels of HBV viremia after loss of HBeAg

More difficult to treat; greater risk of cirrhosis
HBV drug resistance

Treatment with nucleoside analogs (NAs) can lead to prolonged viral suppression (loss of HBV DNA, antigen seroconversion, and, rarely, HBsAg loss and HBsAb appearance) in patients with HBV infection.

However, prolonged therapy may select for mutations in the HBV DNA polymerase gene (including M204V in the YMDD motif), leading to drug resistance.
Laboratory Diagnosis of Resistance

Sequencing
Hybridization (Line Probe Assay)

<table>
<thead>
<tr>
<th>Terminal protein</th>
<th>Spacer</th>
<th>Pol/RT</th>
<th>RNaseH</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>183</td>
<td>349 (rt1)</td>
<td>692 (rt344)</td>
</tr>
</tbody>
</table>

- **F_V_LLAQ**
- **YMDD**

<table>
<thead>
<tr>
<th>I(G)</th>
<th>II(F)</th>
<th>A</th>
<th>C</th>
<th>D</th>
<th>E</th>
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<tr>
<td>LAM resistance</td>
<td>rtA181T/V</td>
<td>rtM204V/I</td>
<td>rtM2041</td>
<td>rtN236T</td>
<td>rtM250I/V</td>
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<tr>
<td>LdT resistance</td>
<td>rtA181T/V</td>
<td>rtM2041</td>
<td>rtN236T</td>
<td>rtM250I/V</td>
<td></td>
</tr>
<tr>
<td>ADV/TDF resistance</td>
<td>rtA181T/V</td>
<td>rtM2041</td>
<td>rtN236T</td>
<td>rtM250I/V</td>
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</tr>
<tr>
<td>ETV resistance</td>
<td>rt169T</td>
<td>rtT1845/A/I/L/G/C/M</td>
<td>rtN236T</td>
<td>rtM250I/V</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>Description</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>Conj. Control</td>
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</tr>
<tr>
<td>2</td>
<td>Amp. Control</td>
<td></td>
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<td></td>
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<tr>
<td>3</td>
<td>L80 WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>V80 Mutant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>I80 Mutant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>V173 WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>G173 WT</td>
<td></td>
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<tr>
<td>8</td>
<td>L173 Mutant</td>
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<tr>
<td>9</td>
<td>L180 WT</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>M 180 Mutant</td>
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<tr>
<td>11</td>
<td>A181 WT</td>
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<td></td>
</tr>
<tr>
<td>12</td>
<td>T181 Mutant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>V181 Mutant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>M204 WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>V204 Mutant</td>
<td></td>
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</tr>
<tr>
<td>16</td>
<td>I204 Mutant</td>
<td></td>
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</tr>
<tr>
<td>17</td>
<td>S204 Mutant</td>
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<td></td>
</tr>
<tr>
<td>18</td>
<td>N236 WT</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>19</td>
<td>T236 Mutant</td>
<td></td>
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</tr>
</tbody>
</table>
Every patient who is HBsAg positive should be tested for anti-HDV IgG antibodies, and if test results are positive, qualitative HDV RNA should be measured.
Hepatitis D: Diagnosis

Acquiring HBV and HDV during the same exposure (HBV/HDV co-infection) is associated with more severe acute hepatitis and HDV is cleared if HBV is cleared.

HDV super-infection in an HBV carrier can manifest as an acute hepatitis and usually results in chronic HDV infection.
Active HDV infection

Although active HDV infection was diagnosed historically by the presence of anti-HDV IgM antibodies, it is now confirmed by the detection of serum HDV RNA with sensitive real-time PCR assay.

Because of the variability of the genome sequence, assays of HDV RNA might produce false-negative results, and testing of anti-HDV IgM antibodies still has a role in patients who test negative for HDV RNA, but have clinical features of HDV-related liver disease.
chronic HDV infection

Qualitative HDV RNA is a marker of viral replication that is positive in chronic infection.

HBV DNA is usually low or negative in chronic HDV infection because HDV suppresses HBV replication.
Quantitative HDV RNA

Serial quantification of HDV RNA is used to determine the response to antiviral treatment.

Quantitative assays, serum concentrations of HDV RNA do not correlate with disease activity or stage of liver fibrosis.

Quantitative HDV RNA is useful to monitor treatment response but this assays are not standardized.
Hepatitis D: Diagnosis

HDV RNA is correlated positively with HBsAg titre and baseline values of both predict response to therapy.

HBsAg is useful to monitor treatment response if quantitative HDV RNA is not available.

Decreasing HBsAg titers often can be surface antigen loss and HDV clearance.
Laboratory diagnosis of hepatitis D Co-infection

HBsAg, HBeAg, and HBV DNA appear in serum during the incubation period in a pattern characteristic of acute hepatitis B.

Both IgM and IgG antibodies to HBcAg appear coincident with the onset of clinical disease.

Evidence of active viral replication often disappears by the peak of illness.

Antibodies to HDAg (anti-HD) develop late in the acute phase of infection and assays for IgM anti-HD and for virion-associated HDV RNA or HDAg in serum are the most reliable markers of acute HDV infection in the presence of high-titer IgM anti-HBc.
The absence of IgM anti-HBc distinguishes superinfection from co-infection.
HDV viremia becomes detectable during the incubation phase and is followed by the appearance of IgM anti-HD and IgG anti-HD during the acute phase.

Markers of HBV replication are usually suppressed during the acute phase and may be difficult to demonstrate active HBV replication at the time of superinfection.

Progression to chronicity is associated with high and persisting levels of IgM and IgG anti-HD.
Hepatitis D: Diagnosis

[Graph showing HBV-HDV coinfection, acute HDV, and chronic HDV superinfection with markers for ALT, IgM, IgG, IgM, IgG, IgG, Anti-HBc, Anti-HD, Anti-HBs, HBsAg, HDV RNA, and HDAg over weeks after exposure.]
Hepatitis D: Diagnosis

Anti-HDV IgG antibody
Positive in all individuals exposed to HDV, and persists long-term, even after viral clearance.

Anti-HDV IgM antibody
Positive in acute infection, negative in past infection but persists in a large proportion of patients with chronic infection. Sometimes used as surrogate marker for HDV replication but not 100% sensitive or specific.

HDV RNA qualitative
Marker of HDV replication. Positive in all patients with chronic infection. Negative in spontaneous or treatment-induced viral clearance.

HDV RNA quantitative
Useful method to predict or monitor treatment response.
Hepatitis D: Diagnosis

**HBsAg qualitative**
Must be positive for HDV infectivity.

**HBsAg quantitative**
Positively correlated with HDV RNA. Might be useful to predict or monitor treatment response, since falling titre heralds HBsAg loss, and hence HDV clearance.

**HBeAg**
Negative in about 85% of patients; associated with detectable anti-HBe.

**HBV DNA quantitative**
Usually negative or low level because suppressed by HDV. Might be increased, especially in patients with detectable HBeAg. Can reactivate after spontaneous or treatment-induced clearance of HDV.

**ALT**
Usually increased but does not correlate well with degree of histological liver damage.
HDV genotyping

HDV genotyping is gaining acceptance as a useful diagnostic test because patients with HDV genotype 1 are at a higher risk of developing end-stage liver disease and have a lower response to treatment with pegylated interferon.
The HDV genotype can be determined by

Restriction fragment length polymorphism analysis (RFLP) of PCR- amplification products, by sequencing,
or, on liver biopsies, by immuno histochemical staining using genome type specific antibodies
Many patients with HBV and HDV have serological evidence of exposure to hepatitis C virus (HCV) about 30% in European cohorts.

In those with triple infection, HDV is the dominant virus because it not only suppresses HBV replication, but also inhibits HCV Replication.

Most patients who are HCV RNA negative have probably cleared HCV infection.