Recent Advances in Diagnosis of Viral Hepatitis A, E, C & G
Definition of hepatitis

- **Inflammation** of the liver

- **Classic hepatitis**?
  Can be caused by a variety of different viruses such as hepatitis A, B, C, D and E

- **Correct diagnosis**
  can only be made by testing patients’ sera for the presence of specific viral antigens and/or anti-viral antibodies & molecular assays
# Viral Hepatitis, Overview

<table>
<thead>
<tr>
<th>Source of virus</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feces</td>
<td>feces</td>
<td>blood/blood-derived body fluids</td>
<td>blood/blood-derived body fluids</td>
<td>blood/blood-derived body fluids</td>
<td>feces</td>
</tr>
<tr>
<td>Route of transmission</td>
<td>fecal-oral</td>
<td>percutaneous permucosal</td>
<td>percutaneous permucosal</td>
<td>percutaneous permucosal</td>
<td>fecal-oral</td>
</tr>
<tr>
<td>Chronic infection</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Prevention</td>
<td>pre/post-exposure immunization</td>
<td>pre/post-exposure immunization</td>
<td>blood donor screening; risk behavior modification</td>
<td>pre/post-exposure immunization; risk behavior modification</td>
<td>ensure safe drinking water</td>
</tr>
</tbody>
</table>
Hepatitis A Virus
Hepatitis A Virus

- Naked RNA virus
- Related to enteroviruses, formerly known as enterovirus 72, now put in its own family: heptovirus
- One stable serotype only
- Difficult to grow in cell culture: primary marmoset cell culture and also in vivo in chimpanzees and marmosets
- 4 genotypes exist, but in practice most of them are group 1
Hepatitis A
Laboratory markers

• **IgM Antibody to Hepatitis A (Anti HAV IgM)**
  – Positive result indicates recent acute HAV infection.
  – Present for 3-6 months after onset.
  – In patients with relapses can persist for more than 12 months.
  – Detected by RIA and ELISA methods

• **Total antibody to Hepatitis A (Anti HAV Ab)**
  – Past infection and immunity to HAV.
  – Individuals given Serum Ig for HAV prophylaxis may test positive for 6 months.

• **Elevated liver enzymes**
  – AST/ALT
  – SGOT/SPOT
**Hepatitis A**

**Laboratory markers**

- **HAV Antigen detection**
  - Detected in stool
  - Expensive, No routine clinical application
  - Detected by IEM and EIA methods

- **HAV RNA**
  - Detected in Blood and Stool specimens
  - No routine clinical application
  - Detected by RT – PCR

- **Cell Culture**
  - Difficult and take up to 4 weeks, not routinely performed
Hepatitis E Virus
Hepatitis E Virus

• Calicivirus-like viruses
• unenveloped RNA virus, 32-34nm in diameter
• +ve stranded RNA genome, 7.6 kb in size.
• very labile and sensitive
• Can only be cultured recently
Diagnosis of hepatitis E

- Acute hepatitis E is diagnosed when the presence of IgM anti-HEV is detected
- Storage of serum samples is acceptable for several days at 4°C,
- Anti-HEV will be preserved at – 20°C,
- A temperature of #-70°C should be preferred when viremia is suspected.
Hepatitis E

- **IgM Antibody to HEV (Anti HEV IgM)**
  - Acute HEV infection. Indicates recent exposure to HEV.
  - Titers decline rapidly during early convalescence.

- **IgG Antibody to HEV (Anti HEV IgG)**
  - Indicates immunity and Old infection.
  - Persists for long periods of time. Sometimes up to > 14 years.
Hepatitis E

Laboratory markers

- **HEV Antigen detection**
  - Detected in Serum and Liver by IFA technique.
  - No routine clinical application.

- **HEV RNA**
  - Detected in Serum and Stool
  - By RT PCR
  - Detected in acute phase faeces in approximately 50% of cases.
Diagnosis of hepatitis E

- To confirm the results of EIA or ELISA tests, Western blot assays to detect IgM and IgG anti-HEV in serum can be used.
- PCR tests for the detection of HEV RNA in serum and stool.
- Immunofluorescent antibody blocking assays to detect antibody to HEV antigen in serum and liver.
- Immune electron microscopy to visualize viral particles in faeces
Hepatitis C virus
Hepatitis C Virus

- Genome resembled that of a flavivirus, positive stranded RNA genome of around 10,000 bases
- 1 single reading frame, structural genes at the 5' end, the non-structural genes at the 3' end.
- Enveloped virus, virion thought to 30-60nm in diameter. Morphological structure remains unknown.
- HCV has been classified into a total of six genotypes (type 1 to 6) on the basis of phylogenetic analysis.
- Genotype 1 and 4 has a poorer prognosis and response to interferon therapy.
- In Hong Kong, genotype 1 accounts for around 67% of cases and genotype 6 around 25%.
HCV Diagnostic assays

**Serology**
- ELISA
  - Anti HCV Ab
- Core Ag
- RIBA

**Biochem**
- Raised SGOT
- Raised SGPT
- Raised S. Bil
- Deranged PT

**Molecular Assays**
- PCR
- RT- PCR
- b-DNA
- TMA
- Genotyping

**Pathological Assays**
- Liver biopsy
- FNAC

**Radiological examination**: Endoscopy, Ultra sonography, CT.
Rational Use of HCV Diagnostic Tests

- Diagnosis
  - Serological assays
  - Qual HCV RNA

- Prognosis
  - Liver histology

- Decision to treat
  - ALT
  - Liver histology
  - Qual HCV RNA

- Treatment duration
  - Viral load

- Treatment

- Response and resistance assessment
  - Qual HCV RNA
  - Viral load
Hepatitis C
Laboratory markers

- **HCV antibody**: generally used to diagnose hepatitis C infection. Not useful in the acute phase as it takes at least 4 weeks after infection before antibody appears.

- **HCV-antigen**: an EIA for HCV antigen is available. It is used in the same capacity as HCV-RNA tests but is much easier to carry out.

- **HCV-RNA**: various techniques are available e.g. PCR and branched DNA. May be used to diagnose HCV infection in the acute phase. However, its main use is in monitoring the response to antiviral therapy.

- **Liver biopsy**: to determine disease activity.
Laboratory Diagnosis

- **Anti HCV antibody**
  - Screening tests
    - Fourth generation ELISA (Antigens: NS3 + NS4 + Core Protein + NS5)
    - Sensitivity – 100% in Immunocompetent and 50 – 95% in Immunosuppressed and Hemodialysis
    - Specificity – 99%

- Supplementary tests
  - RIBA and LIA: Modification of Western Blot.
  - More specific and Less sensitive
  - Anti HCV will not differentiate between acute and chronic HCV infection.
<table>
<thead>
<tr>
<th>Assay</th>
<th>Core</th>
<th>E1</th>
<th>E2/NS1</th>
<th>NS2</th>
<th>NS3</th>
<th>NS4</th>
<th>NS5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ORTHO ELISA</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; generation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c100-3 (a.a.1569-1931)</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; generation</td>
<td>c22-3 (a.a.2-120)</td>
<td></td>
<td></td>
<td></td>
<td>c200 (a.a.1182-1931)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; generation</td>
<td>c22-3 (a.a.2-120)</td>
<td></td>
<td></td>
<td>c200 (a.a.1182-1931)</td>
<td></td>
<td>NS5 (a.a.2054-2995)</td>
<td></td>
</tr>
<tr>
<td><strong>CHIRON SIA</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; generation</td>
<td>c22&lt;sup&gt;p&lt;/sup&gt; (a.a.10-53)</td>
<td></td>
<td>c33c (a.a.1192-1457)</td>
<td>5-1-1p&lt;sup&gt;d&lt;/sup&gt; (a.a.1694-1735)</td>
<td></td>
<td>NS5 (a.a.2054-2995)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c100p&lt;sup&gt;d&lt;/sup&gt; (a.a.1920-1935)</td>
</tr>
<tr>
<td><strong>ABBOTT EIA</strong>&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; generation</td>
<td>HC-34 (a.a.1-150)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HC-31 (a.a.1192-1457 and 1676-1931)</td>
<td>c100-3 (a.a.1569-1931)</td>
</tr>
</tbody>
</table>
Hepatitis C

- **Anti HCV IgM**
  - Positive in 50 – 93% of acute HCV and in 50 – 70% of chronic HCV infection.
  - So cannot be relied upon for acute HCV.
Recombinant immunoblot assay (RIBA)

- Chiron corporation developed a strip immunoassay to resolve true positive from false positive EIA result.

**EIA Antigen (NS3+NS5) + Super oxide Dismutase (hSOD)**

Recombinant

hSOD included on RIBA strip to detect nonspecific antibodies.
Hepatitis C

• **HCV Core antigen detection**
  - Useful for screening in window period.
  - Detected 1 – 2 days after HCV RNA positivity.
  - Low sensitivity.
  - Recently Immune complex dissociation EIA is done
    • Detection limit of antigen – 2 pg / ml
    • 1 pg / ml of HCV Core antigen = 8000 HCV RNA IU / ml
  - Total HCV Core antigen quantification can be used as an alternate to HCV RNA PCR for treatment purposes.
**Prognostic Tests**

- **Viral Load:** patients with high viral load are thought to have a poorer prognosis. Viral load is also used for monitoring response to IFN therapy. A number of commercial and in-house tests are available.

- **Genotyping:** unlike genotypes 2 and 3, genotype 1 and 4 have a worse prognosis overall and respond poorly to interferon therapy. A number of commercial and in-house assays are available.
  - Genotypic methods – DNA sequencing, PCR-hybridization e.g. INNO-LIPA.
  - Serotyping – particularly useful when the patient does not have detectable RNA.
HCV RNA Detection:

HCV RNA in plasma defines active infection

HCV RNA can be detected 1 to 3 weeks post exposure.

Nucleic acid test can be classified in to:

- **Qualitative**: RT-PCR, TMA
- **Quantitative**: bDNA, Real Time PCR
Hepatitis C

- **HCV RNA – Qualitative**
  - Hybridization methods
    - Not useful
    - HCV replicates at relatively low levels and viral genomes may be present only in small amounts.
    - So amplification is necessary.
  - Amplification methods
    - Polymerase chain reaction (RT-PCR)
    - Transcription mediated amplification (TMA)
  - Single Qualitative Positive test – Active viral replication
  - Single Qualitative negative test – Reflects only a viral load below the detection limit of the assay – Repeat test.
Hepatitis C

- **HCV RNA – Quantitative**
  - Target amplification methods – RT-PCR
    - Cut-off: 1000 copies / ml
  - Signal amplification methods – bDNA assay
    - Cut-off: 200,000 copies / ml
  - Real-Time PCR – Best method
  - Quantification is done only for genotypes 1, 4, 5 and 6. For genotypes 2 and 3 only Qualitative analysis is done.
# Commercial available HCV RNA detection assay

<table>
<thead>
<tr>
<th>Test</th>
<th>Method</th>
<th>Manufacturer</th>
<th>Lower limit of detection (IU/ml)</th>
<th>Primary application</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Qualitative</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPLICOR HCV test, version 2.0</td>
<td>RT PCR</td>
<td>Roche</td>
<td>50</td>
<td>Evidence of active infection &amp; response to therapy</td>
</tr>
<tr>
<td>VERSANT HCV RNA qualitative assay</td>
<td>TMA</td>
<td>Bayer</td>
<td>5</td>
<td>-DO-</td>
</tr>
<tr>
<td>Ampliscreen HCV TEST, Version 2.0</td>
<td>RT-PCR</td>
<td>Roche</td>
<td>&lt; 50</td>
<td>Blood Screening</td>
</tr>
<tr>
<td><strong>Quantitative</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPLICOR HCV MONITOR, Version 2.0</td>
<td>RT-PCR</td>
<td>Roche</td>
<td>600</td>
<td>Determination of viral load and length of therapy</td>
</tr>
<tr>
<td>VERSANT HCV NA assay version 3.0</td>
<td>b DNA</td>
<td>Bayer</td>
<td>615</td>
<td>-Do-</td>
</tr>
</tbody>
</table>
Transcription mediated amplification (TMA)

Involves reaction with T7 RNA Polymerase and RT under isothermal condition

Detection limit: as low as 5 copies/ml

Highly Sensitive: 96%

The TMA based VERSANT HCV RNA qualitative assay and procleix HIV/HCV assay was approved by FDA
Transcription-Mediated Amplification

1. Primer 1 (Promoter-Primer) - RNA Target
2. RT - RNA
3. DNA
4. RNA
5. RNAse H Activities
6. DNA
7. Primer 2
8. RNA Pol - RNA
9. Primer 2
10. RT
11. Primers 1 and 2
12. DNA

© Gen - Probe Inc. Reproduced with permission www.chlamydiae.com
Real Time PCR

Syber Green

TaqMan (Primer Probe)

Detection

Amplification Curves
Advantages and Disadvantages of both the chemistry

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Sybr green</th>
<th>Taqman (Primer Probe)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Binds to any double standard product (Nonspecific)</td>
<td>Very specific to the desired fragment</td>
</tr>
<tr>
<td>2</td>
<td>Helpful mainly in qualitative analysis</td>
<td>Helpful in both qualitative and quantitative analysis</td>
</tr>
<tr>
<td>3</td>
<td>Interpretation is little bit tedious</td>
<td>Comparatively simple</td>
</tr>
<tr>
<td>4</td>
<td>Less Expansive</td>
<td>More Expansive</td>
</tr>
</tbody>
</table>
**Guidelines for HCV RNA Testing CDC**

<table>
<thead>
<tr>
<th>Clinical Situation</th>
<th>Test to Use</th>
<th>Interpretation and Comments</th>
</tr>
</thead>
</table>
| Acute infection suspected   | Qualitative PCR or real-time PCR | ❖ Check HCV RNA and HCV ab 4-6 wk after exposure  
❖ Check HCV RNA at 8-12 wk; if positive, consider therapy |
| Chronic infection suspected | Qualitative PCR or real-time PCR | HCV RNA positive: patient is chronically infected  
HCV RNA negative: patient is most likely not infected, but low-level or Intermittent viremia possible. Repeat RNA testing recommended in 6-12 m |
<table>
<thead>
<tr>
<th>Clinical Situation</th>
<th>Test to Use</th>
<th>Interpretation and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV antibody and RNA positive, eligible for treatment</td>
<td>Quantitative tests such as Quantitative qPCR, bDNA, or RT-PCR</td>
<td>&gt;800,000 IU/mL is considered high, more difficult to treat Use same quantitative assay before treatment and measure 4- and 12-wk responses</td>
</tr>
<tr>
<td>Infant born to HCV positive mother; infant still ab. positive at 18 m</td>
<td>Qualitative PCR or real-time PCR</td>
<td>HCV RNA positive: patient is chronically infected HCV RNA negative: patient is most likely not infected, but low-level or intermittent viremia possible. Repeat RNA testing recommended in 6-12 m</td>
</tr>
</tbody>
</table>
Significance of genotyping

- Response of therapy
- Disease severity
- Geographical distribution
- Design of HCV vaccine and therapeutic agents.
Hepatitis C

- **HCV Genotyping**
  - 6 genotypes and numerous subtypes
  
  - Genotyping – Direct sequencing of the NS5B or E1 region.

  - Methods – RFLP, Nested PCR, Reverse Hybridization, Direct sequencing INNO-LIPA, Serological detection of antibodies to genotype specific HCV epitopes.

  - Subtyping is not necessary for treatment purposes.
### Different Methods of Genotyping

<table>
<thead>
<tr>
<th>Method</th>
<th>Region</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type Specific PCR</td>
<td>core</td>
<td>Detect only subtypes.</td>
</tr>
<tr>
<td>RFLP typing</td>
<td>5’NCR</td>
<td>complex, uses 5 to 6 R.E &amp; detects few virus genotypes.</td>
</tr>
<tr>
<td>InnoLiPA</td>
<td>5’NCR</td>
<td>complex, very expensive, do not identify mixed infection.</td>
</tr>
<tr>
<td>Direct sequencing</td>
<td>5’NCR/ Core/ NS5</td>
<td>complex, very expensive,, can not be used in the routine clinical set up.</td>
</tr>
</tbody>
</table>
The INNO-LiPA HCV II:

- Fast, Easy
- Highly specific DNA hybridization test
- Rapid diagnosis of HCV genotypes.
- Expensive
**Restriction Fragment Length Polymorphism (RFLP)**

HCV genotyping typically involves RT-PCR amplification of the 5’UTR.

**Restriction Endonuclease Digestion of the PCR Product**

3 to 5 different enzymes are used

**All six genotypes can be detected**

- Used in routine lab practice.
- Sensitivity 96%
Diagnosis of occult HCV Infection

- **Occult HCV Infection**: HCV RNA undetectable in serum or plasma by current assays, but found in serum, PBMC and/or liver by enhanced molecular tests.

- Very high sensitivity molecular assay like **nested RT-PCR** followed by nucleic acid hybridization technique required.
Candidate for HCV therapy with no contradictions

HCV Genotyping

Genotype 1, 4, 5 & 6

Liver biopsy and viral load measurement to determine benefits of therapy

PEG-IFN+ Ribavirin

Week 12: measure viral load

<2 log drop

≥ 2 log drop but still detectable by qualitative HCV RNA Assay

≥ 2 log drop & undetectable

Non response; stop therapy

Continue therapy

Week 24: Qualitative HCV RNA

+ -

Non response; stop therapy

Continue therapy

Week 48: Qualitative HCV RNA

+ -

Non response; stop therapy

End -of- treatment response; stop therapy

Week 72: Qualitative HCV RNA

+ -

Relapse

Sustain Virological response
Hepatitis G virus
Hepatitis G virus

- A new virus recently identified in Humans.
- Not grown in culture lines
- RNA genome is cloned
- Its role still for debate
- HGV RNA was found in acute, chronic, fulminant hepatitis, hemophiliacs, patients with multiple transfusions
- It resembles HCV in all other aspects
Hepatitis G

- **Agent:** HGV is RNA virus and is similar to HCV but only has 25 amino acids.

- **The virus is named after GB a 34 year-old surgeon** who contracted hepatitis and died from it. His serum was able to infect monkeys and the "GB agent" was passaged in monkeys over the years. **In 1995-96 the virus was identified as a distinct virus different from other human hepatitis viruses (A, B, C, D, E) and was named after the surgeon as HGV.**

- **Three genotypes** of this virus were identified by investigators at Abbott Labs, and termed GB-A, GB-B and GB-C. GB-A and GB-B are likely tamarin viruses; **GB-C can infect humans.**
Hepatitis G

**Clinical Features:**

1: Hepatitis G virus infection is usually "clinically silent" or mild hepatitis and nearly always chronic.

2: Progression to cirrhosis with chronic hepatitis G infection is probably very low.

3: Some studies report chronicity as high as HCV (up to 70%)

4: Nonhepatic manifestations of this virus may well exist.

**Laboratory Diagnosis:**

- ELISA tests results to be confirmed with RIBA
- RT-PCR and Real-time RT-PCR is also available