Molecular Microbiology (part II)

David R. Hillyard MD
Department of Pathology
University of Utah School of Medicine
April 2013
In accordance with ACCME guidelines, any individual in a position to influence and/or control the content of this ASCP CME activity has disclosed all relevant financial relationships within the past 12 months with commercial interests that provide products and/or services related to the content of this CME activity.

The individual below has disclosed the following financial relationship(s) with commercial interest(s):

David Hillyard has received financial compensation in his capacity as a consultant to the following commercial organizations:

- Roche Diagnostics
- Abbott Diagnostics
- ThermoFisher Scientific
- Primera Diagnostics
Thanks to...

- Mathew Bankowski (2011 instructor)
- Ted Schutzbank (2011 instructor)
- Bobby Boyanton (2009 instructor)
- Robert Schlaberg ARUP
- Patricia Slev ARUP
- Brianne Couturier ARUP
- Mel Limson AMP
Hepatitis C Virus (HCV)

- Enveloped RNA virus (Flaviviridae)
- Discovered by reverse genetics as cause Non-A Non-B Hepatitis

- Major types (1-6)
  - Many subtypes
  - Quasispecies

![Pie chart showing the distribution of HCV types: 1a (58%), 1b (22%), 2a (2%), 2b (12%), 3a (4%), 4a (1%)](image)
HCV Genotype Distribution

Copyrighted material

HCV Genome

- Positive Sense RNA
- Single open reading frame

- Gradient of conserved sequence
  - 5’NTR > core > NS5B (Viral load vs Genotyping targets)
    - Viral load tests 5’UTR,
    - genotype tests (5’UTR, core, NS5B) most conserved = best chance amplification, less conserved give better genotype resolution

- Targets for drug therapy
  - NS3/4 Protease (protease inhibitors)
  - NS5B Polymerase (nucleoside/nucleotide & nonnucleoside inhibitors)
Patterns of HCV Infection

Acute HCV $\rightarrow$ Chronic HCV $\rightarrow$ Cirrhosis $\rightarrow$ End Stage Liver Disease & Hepatocellular Carcinoma

Recovery

~15% ↓ ~85%

20-80%
Molecular Testing

- Confirmation of serology for diagnosis
- Determination of genotype (type and duration Rx)
- Therapeutic monitoring (kinetics of response)
- Test of end therapeutic and sustained viral response
Diagnosis

• Serology
  – EIA 3rd generation assays
  – High sensitivity
  – Low specificity
  – Requires confirmation
    • RIBA
    • PCR

• Antigen detection
  – Core antigen
  – Narrows window of detection over 3rd generation EIA

From Patricia Slev
Confirmation HCV EIA

• Recombinant immunoblot assay (RIBA)
  – can be used to confirm low positive anti-HCV screens
  – can distinguish between HCV exposure and a false positive screen
  – cannot discriminate between active and resolved infection
  – should not be used to confirm high anti-HCV screens

✓ – Reagent discontinued 3-13

• HCV RNA testing

✓ – Sensitive **quantitative** or qualitative test
  • Quantitative offers baseline Viral Load
HCV Testing Algorithm

FIGURE 4. Laboratory algorithm for antibody to hepatitis C virus (anti-HCV) testing and result reporting

Screening test for Anti-HCV

Negative* REPORT

OR

Positives* defined by s/co<sup>1</sup> ratios

All positives*

Positives with high<sup>3</sup> s/co ratios

Positives with low<sup>3</sup> s/co ratios

RIBA<sup>2</sup> for anti-HCV

Nucleic acid test for HCV RNA

Negative REPORT

Positive

Indeterminate

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

REPORT

RE
HCV Diagnostic Algorithm

1. High Positive anti-HCV
2. HCV RNA
   - Pos: Currently infected
   - Neg: Never infected
3. anti-HCV by RIBA
   - Pos: Infected, but recovered
   - Neg: ?
4. RIBA “unavailable”
HCV Diagnostic Algorithm

Low Positive anti-HCV

- anti-HCV by RIBA
  - RIBA “unavailable”
  - Pos or indeterminate
    - HCV RNA
      - Pos: Currently infected
      - Neg: Not currently infected, could have recovered

Neg: Never infected (false positive screen)
Therapeutic Modalities

• Interferon
  – Entry, uncoating, mRNA synthesis, HCV antigens onto cell surface, NK cells, interleukins

• Pegylated Interferon
  – More constant, better tolerated effective doses

• Ribavirin
  – Mutagenic (still poorly understood)

• Direct Acting Antivirals (DAA)
  – Many targets
Evolution of HCV Therapy

- Interferon
- Interferon, Ribavirin
- PegInterferon, Ribavirin
- PegInterferon, Ribavirin, Protease inhibitor (triple therapy)

Different testing algorithms for these therapies

Many new Direct Acting Antivirals (DAAs) soon to be approved for protease, polymerase, and other targets. Some therapies do not require Interferon.
Two Approaches to Guided Therapy

- Genotype Guided Therapy
  - Rx some genotypes shorter (GT2,3)
  - Rx other genotypes longer (GT1, 4, 5, 6)

- Response Guided Therapy
  - Rx based on rate of decline in viral levels
HCV Viral Load Testing and Guided Therapy

- Tests measure HCV RNA levels
- Calibrated to WHO international standard
  - Lyophylized type 1 virus from patient (limited availability)
  - I.U. assigned by averaging of HCV copy measurement in many labs
  - Secondary standards allow for calibration of new reagent lots
- Report as log I.U. (reflects broad range of HCV patient VLs)
- Continuous improvement in test sensitivity
  - bDNA
  - NASBA
  - PCR
Patterns of Virologic Response

- **Rapid response**: better outcome, potential shorter treatment
- **“Futility Rules”**: abandon treatment based on response, potential shorter treatment
- **Null response**: worse outcome, potential longer treatment
- **Partial response**: better outcome, potential shorter treatment
- **Relapse**: worse outcome, potential longer treatment

Wks After Start of Therapy

HCV RNA (log_{10} IU/mL)

- **RVR**: rapid viral response
- **EVR**: early viral response
- **EOT**: end of treatment
- **SVR**: sustained virologic response

Undetectable

STOP
HCV Treatment Response Patterns

- **Detailed Response Categories**
  - **RVR**: rapid virologic response – no HCV RNA detected at week 4
  - **eRVR**: extended rapid virologic response – no HCV RNA detected at weeks 4 or 12.
  - **EVR**: early virologic response – virus not detected at week 12
  - **Partial EVR**: - 2 log reduction HCV RNA by week 12
  - **Delayed virologic response**: HCV RNA not detected at week 24 in patients who fail to achieve a complete EVR
  - **End of treatment response (EOT)**: HCV RNA not detected at the end of treatment
  - **Relapse**: reappearance of HCV RNA in patients who were HCV RNA negative at the end of treatment
  - **Sustained virologic response (SVR)**: absence of HCV RNA six months after stopping treatment
Interferon/Ribavirin Therapy

- **HCV Genotype 1**
  - Duration of treatment – 48 weeks
    - Discontinue Tx at 12 weeks if a $2 \log_{10}$ decrease in viral load is not achieved
      - SVR = ~50%
      - Treatment success is <1% if EVR is not achieved
- **HCV Genotypes 2 or 3**
  - Duration of treatment – 24 weeks
    - SVR >75% of patients

NIH consensus guidelines, Management of Hepatitis C:2002
Protease Inhibitors
(current standard of care)

- Peptidomimetics (protease cleavage peptides)
- Telaprevir: rash, anemia, puritis, nausea
- Boceprevir: anemia
- Issues (Resistance and side-effects)
- Test relevant issues
  - Genotype 1 only qualify
  - Stopping rules
    - 100 I.U./ml
    - 1000 I.u./ml
    - “undetected” in assay of appropriate sensitivity
  - Current Viral Load test have sensitivities of ~ 7 I.U./ml

< LOQ does not equal undetected!!!
Response-Guided Therapy Paradigm With BOC + PegIFN/RBV in Tx-Naive Patients

Response-Guided Therapy Paradigm With TVR + PegIFN/RBV in Tx-Naive Patients

### Futility Rules for BOC or TVR + PegIFN/RBV in Tx-Naive Patients

#### BOC\[^{[1,2]}\]

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Criteria</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wk 12</td>
<td>HCV RNA ≥ 100 IU/mL</td>
<td>Discontinue all therapy</td>
</tr>
<tr>
<td>Wk 24</td>
<td>HCV RNA detectable</td>
<td>Discontinue all therapy</td>
</tr>
</tbody>
</table>

#### TVR\[^{[1,3]}\]

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Criteria</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wk 4 or 12</td>
<td>HCV RNA &gt; 1000 IU/mL</td>
<td>Discontinue all therapy</td>
</tr>
<tr>
<td>Wk 24</td>
<td>HCV RNA detectable</td>
<td>Discontinue pegIFN/RBV</td>
</tr>
</tbody>
</table>

Assay should have a lower limit of HCV RNA quantification of ≤ 25 IU/mL and a limit of HCV RNA detection of approximately 10-15 IU/mL.

---

HCV Genotyping Issues

• Accuracy Typing (1 vs some type 6)
• Accuracy Subtyping (1a vs 1b)
• Percentage “no-calls”
  – greater challenge for interrogation of Core or NS5B due to greater sequence variability
• Technical performance
  – throughput, automation, sensitivity
Issues for HCV Genotyping Based on 5’UTR Sequence Analysis

– 5’UTR sequences of genotype 6 isolates other than subtypes 6a and 6b similar to genotype 1.
– 5’UTR sequences within a given genotype are often shared among different subtypes (including 1a, 1b)

Murphy et al 2007 J. Clin Micro Vol. 45, No. 4 p. 1102–1112
Telaprevir Resistance in GT1a vs GT1b

- R155K NS3 protease resistance mutation seen at lower frequency in GT1b vs GT1a
  - Explained by codon usage differences
    - GT1b requires 2 nucleotide changes in arginine codon
    - GT1a uses different codon requiring only 1 change

McCowen Antimicrobial Agents and Chemotherapy, May 2009 p 2129-2132
Sarrazin Gastroenterology 132:1767-1777
HCV Treatment Candidates and Predictors of Poor Outcome

- >18 years
- Antibody & RNA +
- Liver bx (chronic hepatitis), not required
- No Rx contraindications
  - Interferon: hematologic, neuropsychiatric etc
  - Rivavirin: hematologic, reproductive, metabolic
- VL < 400,000 I.U./ml
- Increased Age
- Sex M
- Race African American
- Increased weight
- Liver Fibrosis
- Steatosis
- Insulin resistance
- Alcohol consumption
- All less predictive than IL28
Pharmacogenomics & Hepatitis C

IL28B

rs12979860 C/T  rs8099917 G/T
# IL28B SNPs and Genotypes

<table>
<thead>
<tr>
<th>rs 12979860 C/T</th>
<th>rs 8099917 T/G</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CC</strong> favorable genotype</td>
<td><strong>TT</strong> favorable genotype</td>
</tr>
</tbody>
</table>

- Spontaneous clearance of HCV infection
- Higher SVR to treatment
- Strongest effect in HCV genotype 1

<table>
<thead>
<tr>
<th>CT and TT</th>
<th>TG and GG</th>
</tr>
</thead>
</table>

- Higher risk of hep C chronicity
- Increased risk of treatment failure
- Strongest effect in HCV genotype 1
IL28B & Treatment Induced HCV Clearance
rs 12979860

Copyrighted material

Ge et al. Nature 2009
HCV Key Points

• Diagnose with serology, confirm PCR
• Six types, many subtypes, conserved and variable genome regions
• Value and technical challenge of genotyping
• 5’ UTR genotyping inaccurate for 1a/1b and some 1 vs 6
• Genotype vs Response Guided Therapies
• Stopping rules
• Predictive value of viral response vs host genotype
Hepatitis B Virus (HBV)

- 42 nm
- Hepadnavirus
- Partially dsDNA, ~3200 bp
- Genotypes A-H
- 30-150 day incubation

U.S. Distribution of HBV types

A 7%  
B 27%  
C 51%  
D 15%  
G France, U.S.  
F U.S. Africa  
E sub-Saharan  
D Worldwide  
BC Asia  
A U.S. Europe


From T. Schutzbank
HBsAg Prevalence

- High (≥ 8%)
- Intermediate (2% to 8%)
- Low (< 2%)

**Worldwide**
- 350 million people are chronically infected
- 1-2 million deaths from cirrhosis and hepatocellular carcinoma each year

**In U.S.**
- 1.25 million carriers
- 73,000 new cases each year
- Blood screening by serology and NAAT testing (NAAT not currently required)
HCV Replication

- Replicates in hepatocytes via RNA intermediate
- cccDNA intermediate
- Regeneration of stable cccDNA
- cccDNA stable
- HBV infection cannot be cured by current therapies
HBV Transmission

• Three Routes
  
  *Parenteral route*, contaminated blood. Examples include: post-transfusion, needle sticks, IV drug abuse, hemodialysis

• *Sexual route*, both hetero- and homosexual contact

• *Vertical route*, mother to newborn
HBV Clinical Course
(A Tale of Two Acquisitions)

• Adolescent or adult (I.V. or sexual) U.S.
  – Immediate immune clearance phase
  – Short disease duration
  – Quiescent after anti-HBe conversion (healthy carriers)
  – 3-5% fail to clear

• Early (vertical or close contact) ASIA
  – Prolonged immune tolerance phase
  – Prolonged immune clearance phase
  – Disease progression after HBeAg seroconversion
  – Up to 95% fail to clear
HBV Genome

Double-stranded, enveloped DNA virus
Genome size – 3200 base pairs
Circular DNA molecule – partially
single-stranded
4 open reading frames in the long
(complete) minus strand

DNA → RNA → DNA
Reverse transcriptase lacks proof reading
$10^{11}$ to $10^{13}$ virions daily
All nucleotide positions substituted each
day
Risk resistance and escape mutations
Risk for missed or under-quantified HBV
DNA
### Summary of HBV Biomarkers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg</td>
<td>HBV surface antigen</td>
</tr>
<tr>
<td>Anti-HBs</td>
<td>Antibody to surface antigen</td>
</tr>
<tr>
<td>Anti-HBC</td>
<td>Antibody to core antigen</td>
</tr>
<tr>
<td>Anti-HBC IgM</td>
<td>IgM antibody to core antigen</td>
</tr>
<tr>
<td>HBeAg</td>
<td>Antibody to “e” antigen</td>
</tr>
<tr>
<td>HBV DNA</td>
<td>Viral genome, qual or quant</td>
</tr>
</tbody>
</table>
General Patterns of Response (serology and DNA)

Acute Infection

Chronic Infection
  mild disease

Chronic Infection
  severe disease
Clinical Utility Molecular Tests

- Adjunct to serology for blood screening
- Diagnosis (serology is primary modality)
- Assessment Chronic Disease (along with liver chemistry, biopsy, scanning)
- Therapeutic Monitoring
- Genotype
  - Resistance
  - Escape/expression mutants
  - Genotype (A-H)
• Definition of chronic disease > 20,000 I.U./ml ($10^5$ copies) is arbitrary
  – Cirrhosis and HCC found with lower levels
  – Patients with widely varying levels seen
• Serial monitoring more important than a single arbitrary cutoff
• Low levels ($3$-$5 \log_{10}$ I.U./ml) also associated with progression
  – Setting of cirrhosis
  – HBe-Ag negative
HBV Viral Load Issues

- Broad measuring range (HBV to >10^9 copies/ml)
- Sensitivity (occult HBV)
- Equivalent measurement of Types
- Contamination
  - Viral loads >> log^9 seen
HBV Treatment

- HBIG
- Interferon-α, Peg-Interferon-α
- Nucleosides
  - Lamivudine
  - Adefovir
  - Entecavir
  - Telbivudine
  - Others.....

<table>
<thead>
<tr>
<th>Category</th>
<th>Agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-nucleosides</td>
<td>Lamivudine</td>
</tr>
<tr>
<td></td>
<td>Emtricitabine</td>
</tr>
<tr>
<td></td>
<td>Telbivudine</td>
</tr>
<tr>
<td></td>
<td>Clevudine</td>
</tr>
<tr>
<td>Acyclic phosphonates</td>
<td>Adefovir</td>
</tr>
<tr>
<td></td>
<td>Tenofovir</td>
</tr>
<tr>
<td>Cyclopentane/pentene ring</td>
<td>Entecavir</td>
</tr>
<tr>
<td></td>
<td>Abacavir</td>
</tr>
</tbody>
</table>
Resistance & Genotype Testing
(targets)

HBsAg 10  Clinical Applications  HBsAg 226

TP  Spacer  RT  RH

HBV 1F  HBV 1F  HBV 3R  HBV 2F  HBV 4R  HBV 4R

941 bp

PCR/Sequencing Primer  Sequencing Primer Only

AA 107  MHR  AA 147

HBsAg 10  Conserved NRTI Resistance Domains

Genotype-related Polymorphisms

S. Page D. Hillyard
HBV Resistance Mutations

Drug | RT 26 | RT 323
---|---|---
LMV | L80V/I | V173L, L180M, M204I/V, V207I
TBV | V84M, A181T/V, Q215S | N236T
ADV | V84M, A181T/V, Q215S | N236T
ETV | L169T, S184G, S202I | M250V
TDF | A194T | |
Cross-Resistance Profiles

- Resistance common with early generation drugs (Lamivudine)
- Very low levels seen with Entecavir and Tenofovir
- With breakthrough, choose drug with low cross resistance potential

---

<table>
<thead>
<tr>
<th>Resistance substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVD/LdT-resistant (L180M +/- M204V/I)</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Mutation confers some degree of reduced sensitivity to listed drugs</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Drugs remaining fully active</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Zoulim F & Locarnini S. 2009. Gastroenterol;137:1593
Rational Taxonomic Genotyping

- Genotype C
  - More frequently associated with severe liver disease and HCC than with genotype B
- Genotype B
  - Associated with seroconversion from HBeAg to anti-HBe at younger age than with genotype C
- Genotypes A and B
  - Higher rates of antiviral response and HBeAg loss following peginterferon alfa-2b than with genotypes D and C, respectively

HBV Precore Mutations

- **G1896A**
  - Creates a stop codon in the precore region blocking synthesis of HBeAg
  - Very common in Genotypes B-G, and rare in genotype A
  - G1896 is also a critical base in the stem-loop structure required for encapsidation of pgRNA, pairing with C1858
    - If nt1858 is a U (all genotypes except A), the stem loop is maintained by either G or A1896
    - Genotype A contains a C at 1858, making A1896 defective without a corresponding T to C mutation in 1858.
  - Acquisition of preC G1896A results in lower rates of chronic liver disease

From T. Schutzbank
Basal Core Promoter (BCP) Mutations

- Two mutations occur most frequently in tandem
  - A1762T
  - G1764A
- Found in >80% of all chronic HBV carriers
- Reduced production of HBeAg
Vaccine Escape Mutations

• Cross selection from drug or immune pressure
  – Due to changes in “a” determinant
  – Significance for Chronic HBV Infection unclear
HBV Key Points

• Understanding natural clinical course(s) of HBV
• Genome complexity with immune and drug selection
• Challenge of vast measuring range and contamination for molecular tests
• New expensive drugs with high barrier to resistance
• Correlation with complex serologic markers
Human Papilloma Virus (HPV)

- Most common viral STI
- Incidence \(\sim 6\) million/y; prevalence \(\sim 20\) million
- Lifetime risk \(\sim 50\)-75%
- Clearance 70% at 1 yr, 90% at 2 yrs
HPV - Biology

• Double-stranded, circular DNA, ~8kb
• Oncogenes (E6, E7)
• >100 types (40 infect genital tract)
  – **High risk** (cervical dysplasia/cancer): 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68
  – **Indeterminate risk**
  – **Low risk** (condylomata): 6, 11, 42, 43, 44
• Squamous epithelium
  – Skin, cervix, larynx, oropharynx, anus, esophagus, conjunctiva
Integration

• Chronic infection may lead to integration
  – One or few, fragile site(s) in host genome

• Stabile, high-level expression of E6/E7
  – Fusion to host sequences
  – Proximity to cellular promoters
  – More atypical lesions

• Deletion of large regions of the HPV genome
  – E1, E2
HPV – Pathogenic Spectrum

• **LR HPV**
  – Genital warts, low-grade cervical abnormalities
  – Recurrent respiratory papillomatosis

• **HR HPV**
  – Uterine cervix, vulva, vagina, anus, (penis)
  – Oropharynx (tonsil, base of tongue), esophagus
HPV Causes Cancer in Many Tissues

- Human papillomavirus (HPV) causes >99.7% of all cervical cancers
- HPV also causes cancer in other locations

<table>
<thead>
<tr>
<th>Location</th>
<th>Percent cases due to HPV</th>
<th>number of cases due to HPV worldwide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penis</td>
<td>40%</td>
<td>10,500</td>
</tr>
<tr>
<td>Vulva, vagina</td>
<td>40%</td>
<td>16,000</td>
</tr>
<tr>
<td>Anus</td>
<td>90%</td>
<td>27,400</td>
</tr>
<tr>
<td>Mouth</td>
<td>3%</td>
<td>8,200</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>12%</td>
<td>6,200</td>
</tr>
</tbody>
</table>

Squamous Cervical Precursor Lesions

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSIL</td>
<td>koilocytic atypia (HPV) and/ or CIN1</td>
</tr>
<tr>
<td>HSIL</td>
<td>CIN2/3</td>
</tr>
</tbody>
</table>

J Clin Invest 2006;116:1167-1173
normal cervix → infection → HPV infected cervix → progression → precancer lesions

HPV → viral persistence and progression → precancerous lesion → invasion → cancer

Clearance → mild cytologic &/or histologic abnormalities → regression → clearance
# Natural History of Cervical Precancer

<table>
<thead>
<tr>
<th>Degree of Dysplasia</th>
<th>Regression (%)</th>
<th>Persistence (%)</th>
<th>Progression to CIN3 (%)</th>
<th>Progression to Invasive Cancer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN I</td>
<td>60</td>
<td>30</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>CIN II</td>
<td>40</td>
<td>40</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>CIN III</td>
<td>33</td>
<td>55</td>
<td>N/A</td>
<td>&gt;12*</td>
</tr>
</tbody>
</table>

* Untreated 30% over 30-year period
Treated 1% over 30-year period

Am J Clin Pathol 2012;137:516-542
Cervical Cancer Screening

- Pap test
  - Identifies dysplasia / pre-cancer / cancer
  - High specificity

- HPV test
  - Identifies women at risk
  - High negative predictive value (CIN, cancer)
  - Higher reproducibility

- Combined
  - ASCUS-triage: follow-up interval (≥21y)
  - Co-testing with cytology (≥30y)
Rationale for Genotyping

Copyrighted material
ASC-US Triage

ASC-US

HR HPV-Neg.
- Routine Screening

HR HPV-Pos.
- Referral To Colposcopy

Women > 21
Co-testing
Women > 30

Normal Cytology & HR HPV-pos.

Repeat at 12 Months
- NILM & HPV-neg.
  - Routine Screening
- LSIL or HPV-pos.
  - Colposcopy

HPV Genotyping
- HPV16/18 negative
  - Cotesting At 12 Mo
- HPV16/18 positive
  - Colposcopy
FDA-Cleared HPV Tests

- HC II High-Risk HPV (Qiagen)
- HC II Low-Risk HPV (Qiagen)
  - no role for cervical cancer screening
- Cervista (Invader) HPV High-Risk (Hologic)
- Cervista (Invader) HPV 16/18 (Hologic)
- Cobas HPV Test (L1; HR + 16/18-specific; Roche)
- APTIMA HPV assay (E6/E7 mRNA; Gen-Probe)
- APTIMA HPV Genotyping assay (Gen-Probe)
Hybrid Capture II

- RNA probes representing HPV genome
- RNA Probe-HPV DNA complex (hybrid)
- Capture Ab recognizing hybrid
- AP-conjugated mAb for detection
- **Signal** amplification
- 13 HR types
- 4 ml sample
- No IC
The Analytical Sensitivity of HC2 is 5,000 copies of HPV DNA

The Analytical Sensitivity of PCR methods can be <10 copies of HPV DNA

Adapted from Snijders et al. Journal of Pathology 2003; 201:1-6
Invader

- Probes for 14 HR HPV types
- DNA extraction required
- 2 isothermal reactions
  - Probe and Invader oligo anneal
  - One-base overlap
    in presence of analyte
  - Cleaving of overlapping probes
    ‘cleavase’ reaction
  - Release of 5' flaps plus one nt
  - Rapid on/off; >1 cleavage per target molecule
Invader, Cont.

- Flaps + FRET probe
- Fluorescent signal
- Simultaneous
- Single-well biplex reactions
  - Two flap sequences
  - Two FRET oligos
  - Two fluorophores
- 4 ml sample
- Internal control (gDNA)
Real-Time PCR

- Roche cobas 4800 HPV
- 14 HR types
- Internal control β-globin
- Optional type-specific detection of 16/18
- 1 ml sample
- Automated
E6/E7 mRNA by TMA

- APTIMA HPV assay
- Detection of E6/E7 mRNA
- 14 HR types
- Single tube
  - Target capture
  - Target amplification (TMA)
  - Detection (hybridization protection assay)
- Internal control
- Automated
- Separate genotyping assay (HPV 16 vs. 18/45)
HPV Vaccination

- Two vaccines currently available
  - Gardasil (Merck)
    - Quadravalent: HPV 6,11,16,18
    - Duration – at least 5 years
  - Cervarix
    - Bivalent – HPV 16,18
    - Duration – at least 5 years
- Neither vaccine provides significant cross protection against other high risk HPV types
HPV Key Points

- Negative predictive value of molecular testing
- Challenge of clinical validation for “best test”
- Importance of “sweet spot” for molecular sensitivity
- Importance of typing
- Vaccine importance and its immediate non-impact on molecular testing algorithms
Enterovirus

- Genus Enterovirus has 10 species
  - Human EV-A, B, C, D, and Poliovirus
  - Simian EV-A, Bovine EV, Porcine EV-B
- New isolates called “enteroviruses” followed by #...first being EV68
- All major groups defined by multiple full sequences
- RNA genome
Enteroviruses
Spectrum of Illness

• Asymptomatic
• Non-specific febrile illness
• Hand-foot-mouth disease
• Acute hemorrhagic conjunctivitis
• Myocarditis
• Hepatitis
• “Sepsis” syndrome
• Central nervous system infections
  aseptic meningitis chronic enteroviral meningoencephalitis
  encephalitis paralytic myelitis
Molecular Targets

- 5’ UTR for detection
- VP1-3 for typing (sequence based)
Burden of Disease (EV)

- Among most common human viruses
  - Estimated 50 million U.S., 1 Billion worldwide
- < 1% cause significant illness
- 30-50,000 hospitalizations/year U.S.
- Sporadic circulation: Cox B5, ECHO 6, 9, 30 (2003-2005)
- Epidemic circulation: Cox A9, B3, B4, EV 71
Pathogenesis and Clinical

• Primary site infection: epithelial cells of gut and respiratory tract, lymphoid cells of small intestine
• Disease due to
  – Cytopathic (CNS)
  – Host immune response (exanthems, myocarditis)
• Incubation times 7-14 days (2-35)
• Link to disease often tenuous
  – Occult infection and prolonged excretion are common
Viral Detection

- febrile
- irritable
- stiff neck
- “just doesn’t look right”

? diagnosis

- simple childhood infection
- self resolving meningitis
- life threatening meningitis (bacteria, fungus, herpes virus)
- other

Enterovirus - most common cause aseptic meningitis (usually with a good outcome)

Test Spinal Fluid → culture (slow & insensitive) → negative result → look for other causes
(molecular test (rapid & sensitive) → positive result → child usually goes home (~3 day decreased hospital stay > $4,500)
Enterovirus Testing by PCR

- Has been demonstrated to be superior to viral culture in multiple studies
  - Sensitivity 98.6%, Specificity 99.4%
- Blood and CSF are equally good specimens for PCR testing
- EV testing by PCR routine for febrile children and all children with CSF pleocytosis
  - Turn around time is important and should be < 24 hours for maximum utility in clinical decision making
EV PCR is Cost-Effective

- Positive EV PCR results have been associated with:
  - Fewer ancillary tests (Ramers, 2000, Archimbaud 2009)
  - Decrease antibiotic use (Ramers, 2000, Robinson 2002, Archimbaud, 2009)
  - Decreased hospital length of stay (Ramers, 2000, Stellrecht 2002, Archimbaud, 2009)
  - Decreased hospital charges (Robinson, 2002)
Optimal Format for Enterovirus RNA Testing?

• RT-PCR
  – gel
  – ELISA (10-100 fold post-PCR signal boost)

• Real-time RT-PCR
  – TaqMan
  – Hyb-probes
  – NASBA

• Optimal
  – Very Rapid TAT
  – STAT lab compatible?
Example Rapid Platform

- Cephiad GeneExpert
- FDA approved EV PCR assay
- TaqMan chemistry
  - Mixed probe sequence
- Suitable for Stat Labs
- Results 2.5 hours
Enterovirus 5’ UTR Most Conserved but Still Hypervariable (limited target sites and risk non-detection)

Copyrighted material
RNA Virus Genomes are Polymorphic

- 2 distinct melting temps 53°C and 61°C
- 6 samples (18%) seen only by melt

Unforeseen polymorphism?  no CT
Problem Samples have SNPs in Probe Region

- WT sequence - CTTTTGGGTG
- Sequence 1 - CTTTTGGGTG
- Sequence 2 - CTTTTGGGAGT
Parechovirus Overview

• Discovered 1956
  – Summer diarrhea outbreak
  – “echovirus 22,23”
  – Sequenced as distinct genus 1992
  – Types 1-16 (9 fully sequenced)

• Disease of young children
  – 68-73% infections under age 1
  – 1 case in literature of infection > 10 yrs
    • HPeV1 (6.6 mos)
    • Hpev3 (1.3 mos)

Joki-Korpela and Hyypia CID 1998;27 (July)
Parechovirus Disease

• Spectrum
  – Neonatal Sepsis
  – Meningitis/Encephalitis
    • Main indications for clinical testing!!
  – GI, Respiratory Disease correlates (lacking definitive association)

• Significant strain differences
  – HPeV3 has higher association with neonatal disease
    • Lower adult seroprevalence (maternal antibody)
    • Emergence from common source within last 2 decades
    • Alternative cell receptor (no RGD motif in VP1*)

• Prevalence may be comparable to EV

CNS Neurotropism

- Well established association for EV
- Preliminary studies suggest similar association for HPeV
Challenges

• Fewer clinical labs now attempt virus isolation for suspected EV disease
  – Decreased opportunity for virus characterization
  – Few labs test for parechoviruses, cardioviruses
• Need for quality “targeted picornavirus” PCR detection assays
• Difficulty of molecular typing (variable viral sequence domains)
• Combined EV-HPeV detection, HPeV typing?
Enterovirus Key Points

• Clinical algorithm and importance of negative predictive value
• Genome variability and implications for detection and typing
• Importance of rapid testing
Herpes Simplex Virus

• Primarily infects mouth and/or genitalia
• Two Strains
  – HSV-1
  – HSV-2
• Both viruses cause painful vesicles on the skin at the site of inoculation.
  – HSV1 is usually associated with oro-facial lesions
  – HSV2 is usually associated with genital lesions
Herpes Simples Encephalitis

• most common identifiable cause of human acquired sporadic encephalitis
• HSV-1  90%     HSV-2  10%  (age dependent)
  – primary infection, reactivation, reinfection
• Pathology
  – acute inflammation, hemorrhage, necrosis
• Location
  – typically temporal lobes and orbital surfaces of frontal lobes
  – any region of brain
HSV Encephalitis Clinical Course

• Prodome
  – Fever, headache, nausea

• Neurological symptoms
  – Seizures, clouding of consciousness, aphasia, olfactory changes, behavioral changes

• Typical Course
  – Rapid progression
  – Brain edema (brain stem compression)
  – Death (7-10 days) 70% mortality with placebo Rx severe neuro damage in survivors.
Clinical Applications of HSV PCR

• Rapid diagnosis of HSV encephalitis
  – >95% sensitive in adults
  – ~70 – 100% sensitive in neonates
  – Culture positive in < 5%
  – Essential for appropriate use of antimicrobial therapy
  – Monitor response to antiviral therapy
HSV Quantification?

• 16 Patients treated with acyclovir
• quantitative testing done within 4 days of presentation
• values range from 25,000 to 18,000,000 copies/ml
• values above 100,000 copies/ml had worse outcomes

Clinical Applications of HSV PCR

• Detect subclinical shedding of HSV during pregnancy
  – ~70% of neonatal HSV infections result from asymptomatic shedding by the mother
  – PCR considerably more sensitive than culture for detecting HSV in vaginal secretions
Clinical Applications of HSV PCR

• Replacement for culture in more routine situations
  – HSV skin and genital lesions
    Rapid TAT
  Greater sensitivity than culture
HSV Key Points

• Importance of CNS testing in young and old
• Tests may be negative very early in infection and virus may persist in early phases of successful treatment
• Critical need for sensitive testing
• Need for internal controls
• Need to confirm low positive PCR results?
Varicella Zoster Virus

- Two clinical entities
  - Varicella (Chicken pox)
  - Herpes Zoster (Shingles)

- CNS infection vs HSV
  - Less frequent
  - Less severe clinical course
Bordetella pertussis

- Severe, debilitating illness (“100 day cough”)
- Highest morbidity and mortality in infants
- Despite high vaccine coverage, remains a serious public health problem
- Outbreaks continue
  - Overall high vaccination rates
  - General and regional vaccine compliance issues
Bordetella parapertussis

- Pertussis-like illness but more mild
  - Prolonged cough, paroxysms, whoop, vomiting
- Does not produce pertussis toxin
- More severe in young children
- Death is rare
- Co-infection with B. pertussis and B. parapertussis occurs
- No case definition
- Not reportable
Timing and Specimen Collection for PCR

- Best sensitivity within first 3 weeks of cough
- Not recommended beyond 5th day of antibiotics
  - DNA detected when cultures won’t grow
- Swab or aspirate posterior nasopharynx
- Calcium alginate swabs not acceptable (PCR inhibition)
- Avoid contamination
  - Vaccination areas (some contain B. pertussis DNA)
  - Bleach clinic environments, glove during collection
  - Use solid or semi-solid transport media or dry swab

www.cdc.gov/pertussis/clinical/diagnostic-testing/diagnosis-PCR-bestpractices.html
Culture

• “Gold Standard”
  – Public health labs
  – Susceptibility and typing

• High specificity, low sensitivity
  – Best within 2 weeks onset of cough
  – Young patients
  – Unvaccinated

• Slow
  – Incubation time 4-10 days
Molecular Infectious Disease
(Bordetella pertussis and Bordetella parapertussis)

• Gene targets detecting *B. pertussis and other Bordetella spp.*
  – **IS481** (*B. pertussis* [50-100+ copies/cell], *B. holmesii*, [8-10 copies/cell], and some strains of *B. bronchiseptica)*
  – **IS1001** (*B. parapertussis* and *B. holmesii*)
  – Pertactin gene (*Some strains of B. bronchiseptica)*
  – Chromosomal gene target, adenylate cyclase toxin gene (*cyaA*) is also not specific for *B. pertussis*

Molecular Infectious Disease
(Bordetella pertussis and Bordetella parapertussis)

• Chromosomal gene targets specific for B. pertussis
  – Promoter region (PTp1 and PTp2)
  – DNA region upstream of the porin gene
  – Repeated insertion sequences (IS)
  – Pertussis toxin promoter (ptxA-Pr)*
    • Note: The pertussis toxin operon is present in B. pertussis, B. parapertussis, and B. bronchiseptica
  – ACT gene
  – BP3385, BP283, and BP485

Test Interpretation

- Tests not standardized (methods or cutoffs)
- High cycle threshold (Ct) value interpretation
  - Infection vs Contamination?
- Reporting options
  - Detected (positive), Indeterminate, Equivocal
- Single target IS481 tests most vulnerable to false positives
  - High copy number in B. pertussis
  - Lower quantities in B. holmesii and B. bronchiseptica
- Multi-target assays preferred

www.cdc.gov/pertussis/clinical/diagnostic-testing/diagnosis-pcr-bestpractices.html
CDC Assay Species Discrimination

<table>
<thead>
<tr>
<th>Species</th>
<th>ptxS1</th>
<th>IS481</th>
<th>hIS1001</th>
<th>pIS1001</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. pertussis</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. parapertussis</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>B. pertussis and B. parapertussis</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>B. holmesii</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

- Interpret PCR results along with the clinical symptoms and epidemiological information
- Determine the clinically relevant cut-off value for all targets
- Use indeterminate as a result for multi-copy target for IS481, not ptxS1
Bordetella Key Points

- Pertussis vs parapertussis clinical
- Critical issues of sample collection and contamination
- IS481 is multicopy and not specific for B. pertussis
- Monoplex vs muliplex testing (sensitive and specific)
- Conservative PCR Ct interpretation
Clostridium difficile

- Anaerobic, gram-positive rod
- Toxigenic vs. non-toxigenic strains
- Spores
  - Resistant to heat, acid, antibiotic
- Vegetative forms
  - Toxin producing
- Risk factors
  - Antibiotic use
  - Hospitalization
  - Severe illness
  - Gastric acid suppression
Symptoms

• Case definition of CDI
  – Symptoms (diarrhea x3/24h)
  – Stool test or pseudomembranous colitis
• Colitis with watery diarrhea
• Onset classically during/after antibiotic therapy
• Median onset 2-3 days after colonization
• Nosocomial vs. community acquired
• Recurrence in 10-25% (relapse vs re-infection)
Diagnosis

• Indication for testing
  – 2 days of significant diarrhea (3+ stools/d)
  – 1 day of 10-15 stools/d, fever/nocturnal diarrhea
  – Exception: ileus

• Recurrence: same as initial episode

• No indication
  – Asymptomatic, i.e. formed stool specimens, unless ileus is suspected
  – No test of cure

Infect Control Hosp Epidemiol 2010; 31(5)
Toxins

- Potent exotoxins
  - Receptors on intestinal epithelial cells
  - Mucosal injury, fluid secretion, inflammation
  - Toxin A ("enterotoxin")
  - Toxin B ("cytotoxin")

- Stool toxin levels proportional to disease severity

- PaLoc
  - Includes tcdA, tdcB

- Most strains: toxins A and B
  - Variant toxin expression

Nature. 2010 Oct 7;467:711
J Clin Microbiol 2000; 38:1696
J Clin Microbiol 2002;40:2079
Ann Intern Med 2001;135:434
The Hyper-virulent and Epidemic Strain

- Molecular Classification:
  - Toxinotype III
  - BI by REA (REA Analysis)
  - NAP1 by PFGE
  - 027 by ribotyping

- Fluoroquinolone Resistant

- Increased Disease Severity
  - Increased morbidity / mortality

- Virulence Factors
  - Hyper Spore Production
  - Binary toxin \(cdtB\)
  - \(tcDC\) 18 bp deletion
    - ~20-fold increased amount of toxins A and B released

From Bobby L. Boyanton

http://www.cdc.gov/ncidod/dhqp/id_Cdiff_data.html
Toxigenic *Clostridium difficile*

Pathogenicity Locus (PaLoc)

19.6 Kb

- **tcdD**: Positive Regulator
- **tcdB**: Cytotoxin
- **tcdE**: Holin Function?
- **tcdA**: Enterotoxin
- **tcdC**: Negative Regulator

18 bp deletion


Slide created by Bobby Boyanton, M.D.
Diagnosis

- Toxigenic culture
  - Stool ➔ culture ➔ isolate ➔ cytotoxin detection
- Cytotoxin assay
  - Stool ➔ cytotoxin detection
- Toxin A/B EIA
  - Stool ➔ toxin EIA
- GDH + toxin detection
  - Stool ➔ GDH ➔ if positive: toxin detection
- NAAT
  - Stool ➔ NAAT
Nucleic Acid Amplification Tests

- Methodologies
  - Real-time PCR
  - Isothermal amplification (LAMP and others)
- High sensitivity
- High specificity
- Rapid
  - Treatment, infection control
- More expensive, less experience needed
- Limited PPV with prevalence <10%

Example Algorithm

A)

GDH assay

positive

Toxin A/B assay

positive =
Positive for CDI

negative

PCR assay

positive =
Positive for CDI

negative =
Negative for CDI

negative =
Negative for CDI

ASM: A Practical Guidance Document for C. difficile Toxin Laboratory Testing, 8/24/2010
SHEA, IDSA Guidelines

- Test only diarrheal stool (exception: ileus)
- Don’t test if asymptomatic (test of cure)
- Culture most sensitive, not clinically practical
  - Reference test if performed by experienced lab
- Toxin A/B EIA suboptimal (rapid, less sensitive)
- GDH screening + cytotoxicity/culture
  - Sensitivity varies by kit, interim recommendation
- NAAT rapid, sensitive, specific
  - More data on utility necessary
- No repeat testing during same episode

Infect Control Hosp Epidemiol;31(5):431-55
Mycobacteria

• Drivers for molecular testing
  – Rising rates of resistance
  – Clinical and cost impact of slow Dx
  – (AFB) smear inexpensive but insensitive
  – Can’t differentiate M. tuberculosis from other mycobacteria

• 2009 CDC recommendation
  – NAATs should be performed on at least 1 respiratory sample from patients with signs and symptoms of pulmonary M. tuberculosis colonization or infection .....if it would alter case management or infection control
AFB smear with the added value of NAAT testing

1. Greater positive predictive value (>95%) with AFB smear-positive specimens in settings in which nontuberculous mycobacteria are common.

2. Confirm rapidly the presence of *M. tuberculosis* in 50%--80% of AFB smear-negative, culture-positive specimens.

3. Compared with culture, NAAT tests can detect the presence of *M. tuberculosis* bacteria in a specimen weeks earlier than culture for 80%--90% of patients suspected to have pulmonary TB whose TB is ultimately confirmed by culture.

4. Can impact patient care and TB control efforts
   
   Avoiding unnecessary contact investigations or respiratory isolation for patients whose AFB smear-positive specimens do not contain *M. tuberculosis*. 

---

*Updated Guidelines for the Use of Nucleic Acid Amplification Tests in the Diagnosis of Tuberculosis*

*MMWR* - January 16, 2009 / 58(01);7-10
Test Options

- MTB screening
  - PPD
  - Quantiferon TB Gold (IFN gamma)

- Laboratory testing
  - Nucleic acid probes
  - Nucleic acid amplification
  - Culture
    - Solid (Middlebrook, Lowenstein-Jensen)
    - Liquid (Automated)
  - Biochemical testing

- Susceptibility testing
  - INH, streptomycin, rifampin, ethambutol, ethionamide, pyrazinamide

Gilligan, et.al. 2003 (3rd Ed.). *Cases in Medical Microbiology and Infectious Diseases*. ASM Press.
Infectious Disease
(Mycobacterium sp.)

• FDA cleared assays
  – Polymerase chain reaction (PCR)
  – Transcription medicated amplification (TMA)
  – Direct culture hybridization probes (AccuProbe)
    – Mycobacterium avium
    – Mycobacterium intracellulare
    – Mycobacterium avium complex
    – Mycobacterium gordonae
    – Mycobacterium kansasii
    – Mycobacterium tuberculosis complex
      » M. tuberculosis, M. bovis, M. bovis BCG, M. africanum, M. microti, and M. canetti

• In-house Developed Assays
  – Conventional PCR
  – Real-time PCR
  – NASBA
Malaria

- Gold Standard is Giemsa-stained Thick & Thin smears
  - Thick smears typically used for screening
  - Thin smears used for speciation; determining percent parasitemia
- Rapid antigen tests, PCR and serology also used
  - Problems – previously positive give false positive serology; Rapid antigen test will not give parasitemia
  - Rapid antigen tests appropriate for small labs as a screening tool
Blood Smears

• Thick smears
  – Air dried and placed in hypotonic stain (Giemsa stain)
  – Screen for presence of parasites
  – Limit of detection is ~ 50,000 parasites/ml

• Thin smears
  – Fixed in methanol before staining to preserve morphology of RBC’s and parasites
  – Requires skilled technologist for accurate analysis
Molecular Plasmodium Detection and Speciation

• Detect four malarial parasites
  – *Plasmodium vivax*
  – *Plasmodium falciparum*
  – *Plasmodium malariae*
  – *Plasmodium ovale*

• Direct detection from blood specimen

• High sensitivity and specificity

• Labor savings over reading thick and thin blood smears
Methods Plasmodium Detection and Speciation

• PCR
  – Many formats
  – Real-time PCR (18S rRNA) with species specific probes
• LAMP
• QT-NASBA (18S rRNA)
• Microarrays
• Flow cytometry
• Mass Spectrometry
• Sequencing and Pyrosequencing
• Gene detection for antimalarial resistance
  – Chloroquine and Mefloquine

Plasmodium sp. Real-time PCR Test Performance – Realtime PCR vs. Microscopic*
