CMV Diagnostic Strategies: Current and Future

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Faculty/Presenter Disclosure

Relationships with commercial interests:

- **Grants/Research Support:** Qvella, bioMerieux
- **Speakers Bureau/Honoraria:** Merck, Sunovion
- **Advisory Board:** Merck, Pfizer, Qvella, Microbix, Roche
Mitigating Potential Bias

No financial or other support has been received from any commercial entity for the preparation or presentation of this talk.
Learning Objectives

At the end of this presentation, participants will be able to:

- Cite current tests available for the diagnosis and management of CMV disease in SOT and HSCT patients including resistance testing
- Interpret results of resistance testing
- Recognize future testing options
CYTOMEGALOVIRUS

- Betaherpesvirus:
  - ds DNA
  - Icosahedral capsid
  - Lipid envelope

- Establishes latency:
  - "Once infected always infected"

- Prevalence:
  - Species specific
  - 40-70% adult pop’n
Cytomegalovirus Latency

Cytomegalovirus Latency

Human CMV Virion Structure

Available Tests for Detection, Diagnosis, & Monitoring of CMV

- CMV serology (IgG, IgM, and avidity testing)
- Direct detection – EM, In-situ hybridization, Antigenemia
- Culture: Tube and Shell vial
- Molecular – Quantitative and Qualitative detection of CMV DNA
- Antiviral Resistance testing - sequencing the UL97 and/or UL54 (polymerase) genes directly from a plasma specimen (genotypic); plaque reduction assays (phenotypic)
CMV Diagnosis and Monitoring in Transplantation

Antigenemia (Mid/Late 1990s)

Molecular (Early/Mid 2000s)
<table>
<thead>
<tr>
<th>CMV antigenemia</th>
<th># specimens</th>
<th>CMV PCR</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV antigenemia</td>
<td># samples</td>
<td></td>
<td># samples</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td># samples</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
<td>44</td>
<td>30 (68%)</td>
<td>14 (32%)</td>
<td></td>
</tr>
<tr>
<td>0-2 pos cells/10^5</td>
<td>37</td>
<td>2 (5%)</td>
<td>35 (95%)</td>
<td></td>
</tr>
<tr>
<td>3-5 pos cells/10^5</td>
<td>14</td>
<td>0</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>&gt;5 pos cells/10^5</td>
<td>16</td>
<td>0</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>111</td>
<td>32</td>
<td>79</td>
<td></td>
</tr>
</tbody>
</table>
CMV Quantification by PCR & Antigenemia

log_{10} HCMV DNA copies / 2.10^5 PBLs

log_{10} pp65 positive cells / 2.10^5 PBLs

P < 0.0001

CMV Quantification by PCR & Antigenemia

Mazzulli, T et al. Unpublished
Linearity of CMV PCR

Linearity and Limit of Detection of CMV PCR in copies/mL (plasma extracted in EasyMag, Astra reagent)

Log (expected)

Log (obtained)

Log (obtained copies/mL)

Plasma samples in serial dilutions

Mazzulli, T et al. Unpublished
Clinical Utility of CMV DNA Testing

Clinicians may use a CMV DNA test for determining:

1. Likelihood that a symptomatic patient has active CMV disease
2. Likelihood that an asymptomatic patient will develop active disease
3. A patient’s response to therapy
4. The risk of developing relapsed infection
5. The appropriate time to discontinue therapy
Performance and Interpretation of CMV DNA Testing

- Standardization – Commercial vs In-house and use of WHO International Standard
- Sample type and stability – Whole blood, serum, plasma, buffy coat
  - Viral load may be ~1 to 2 log_{10} (up to 100-fold) higher in whole blood than plasma in same patient
  - CMV DNA stable up to 14 days in plasma at 4°C
- Analytical test characteristics – LOD, LOQ, Precision, Accuracy, Specificity, (Sensitivity?)
  - clinical significance of low levels of CMV DNA (100–500 copies/mL) may be difficult to interpret

Performance and Interpretation of CMV DNA Testing

Determining the threshold for treatment:

- e.g. viral load value of 2000–5000 copies/mL correlated with the development of end organ disease in liver transplant using the Cobas Amplicor Monitor Assay¹
- Some studies suggest both the viral load value and the rate of change in viral load are important predictors of the development of active disease²

Performance and Interpretation of CMV DNA Testing

Assay variability:

- No clear difference exists between results in a patient in whom the viral load value has increased from 200 copies/mL to 500 copies/mL.

- Viral load changes of $0.5 \log_{10}$ copies/mL in HIV and other viruses are thought to represent biologically important changes in viral replication – does this apply to CMV DNA?

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 18 to 22 October 2010

Collaborative Study to Evaluate the Proposed 1st WHO International Standard for Human Cytomegalovirus (HCMV) for Nucleic Acid Amplification (NAT)-Based Assays

Jacqueline F. Fryer\textsuperscript{1,3}, Alan B. Heath\textsuperscript{2}, Rob Anderson\textsuperscript{1}, Philip D. Minor\textsuperscript{1} and the Collaborative Study Group *

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National Institute for Biological Standards and Control,
South Mimms, Potters Bar, Herts, EN6 3QG, UK

\textsuperscript{3} Study Coordinator; Tel +44 1707 641000, Fax +44 1707 641050,
E-mail Jacqueline.Fryer@nibsc.hpa.org.uk

* See Appendix 1

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Overall Mean Estimates and Inter-Laboratory Variation (log10 ‘copies/mL’ for quantitative or ‘NAT-detectable units/mL’ for qualitative assays)

1st WHO International Standard for HCMV:
- Whole virus preparation of ‘Merlin’ HCMV strain
- $5 \times 10^6$ copies/mL ($6.7 \log_{10}$)
  - Individual lab mean estimates ranged from 5.4 to $7.5 \log_{10}$ copies/mL
- Uncertainty of 0.23%
- Stable for up to 8 months (freeze/thaw stable)
Are We There Yet? Impact of the First International Standard for Cytomegalovirus DNA on the Harmonization of Results Reported on Plasma Samples

Jutta K. Preiksaitis,1 Randall T. Hayden,2 Yupin Tong,1 Xiaoli L. Pang,3 Jacqueline F. Fryer,4 Alan B. Heath,4 Linda Cook,5 Astrid K. Petrich,6 Brian Yu,7 and Angela M. Caliendo8
## Characteristics of CMV PCR Assays

<table>
<thead>
<tr>
<th>Test</th>
<th>Lab1</th>
<th>Lab1</th>
<th>Lab2</th>
<th>Lab3</th>
<th>Lab3</th>
<th>Lab3</th>
<th>Lab4</th>
<th>Lab5</th>
<th>Lab5</th>
<th>Lab6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay characteristic</td>
<td>MultiCode-RTx CMV</td>
<td>COBAS AmpliPrep/</td>
<td>RealStar CMV PCR Kit 1.0</td>
<td>RealStar CMV PCR Kit 1.0</td>
<td>CMV LC-PCR</td>
<td>arus CMV RG PCR Kit</td>
<td>Quantitative TaqMan PCR</td>
<td>Abbott RealTime CMV</td>
<td>arus CMV RG PCR Kit</td>
<td>Simplexa CMV Kit</td>
</tr>
<tr>
<td>Manufacturer</td>
<td>LumineX Corporation, Madison, WI</td>
<td>Roche Molecular Systems, Pleasanton, CA</td>
<td>Altana Diagnostics, Hamburg, Germany</td>
<td>Altana Diagnostics, Hamburg, Germany</td>
<td>LDT, Edmonton Canada</td>
<td>Qiagen, Hilden, Germany</td>
<td>LDT Seattle, WA</td>
<td>Abbott Molecular, Des Plaines, IL</td>
<td>Qiagen, Hilden, Germany</td>
<td>Diagnostic, Inc, Cypress, CA</td>
</tr>
<tr>
<td>Registration status</td>
<td>Commercial, ASR</td>
<td>Commercial, FDA-approved CE-marked</td>
<td>Commercial, CE marked</td>
<td>Commercial, CE marked</td>
<td>Commercial, CE marked</td>
<td>Commercial, CE marked</td>
<td>Commercial, CE marked</td>
<td>Commercial, CE approved CE marked</td>
<td>Commercial, CE marked</td>
<td>Commercial, CE marked</td>
</tr>
<tr>
<td>Test calibrators</td>
<td>LD amplicon</td>
<td>MP Plasmid</td>
<td>MP amplicon</td>
<td>MP amplicon</td>
<td>LD Plasmid</td>
<td>MP Plasmid</td>
<td>MP Plasmid</td>
<td>MP amplicon</td>
<td>MP amplicon</td>
<td>MP amplicon</td>
</tr>
<tr>
<td>Gene Target</td>
<td>DNA Polymerase (UL54)</td>
<td>DNA polymerase (UL54)</td>
<td>confidential</td>
<td>confidential</td>
<td>Glycoprotein B (UL59)</td>
<td>MIE</td>
<td>Glycoprotein B (UL59) and IE (UL123)</td>
<td>UL34 UL80.5</td>
<td>MIE</td>
<td>UL83</td>
</tr>
<tr>
<td>Amplicon size(s)x(bp)</td>
<td>52</td>
<td>340</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>254</td>
<td>105</td>
<td>64 (UL59), 76 (UL123)</td>
<td>95 (UL80.5), 105 (UL34)</td>
<td>105</td>
<td>96</td>
</tr>
<tr>
<td>Probe Chemistry</td>
<td>FRET labeled primers</td>
<td>TaqMan</td>
<td>TaqMan</td>
<td>TaqMan</td>
<td>FRET</td>
<td>TaqMan</td>
<td>TaqMan</td>
<td>TaqMan</td>
<td>TaqMan</td>
<td>TaqMan</td>
</tr>
<tr>
<td>Extraction method</td>
<td>Qiagen E21 Advanced XL</td>
<td>Roche COBAS AmpliPrep</td>
<td>King Fisher FLex</td>
<td>Qiagen QIAcube</td>
<td>Qiagen QIAcube</td>
<td>Qiagen EZ1 Advanced</td>
<td>Roche MagNa Pure 96</td>
<td>Abbott m2000</td>
<td>Qiagen EZ1 Advanced XL</td>
<td>Qiagen EZQ Virus Mini Kit</td>
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<tr>
<td>Extraction Kits</td>
<td>Qiagen E21 Advanced XL</td>
<td>Roche COBAS AmpliPrep</td>
<td>King Fisher MagMAX Viral Isolation Kit</td>
<td>Qiagen DNA Mini Kit</td>
<td>Qiagen DNA Mini Kit</td>
<td>Qiagen EZ1 DSP Virus Kit</td>
<td>Roche MagNa Pure 96 DNA and Viral NA Small Volume Kit</td>
<td>Abbott Sample Preparation System DNA Kit</td>
<td>Qiagen EZQ Virus Mini Kit v2.0</td>
<td></td>
</tr>
<tr>
<td>Plasma input volume</td>
<td>200 µL</td>
<td>500 µL</td>
<td>100 µL</td>
<td>200 µL</td>
<td>200 µL</td>
<td>400 µL</td>
<td>200 µL</td>
<td>600 µL</td>
<td>200 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>LOD, log_{10} IU/mL</td>
<td>3.35</td>
<td>2.14</td>
<td>2.40</td>
<td>1.61</td>
<td>1.64</td>
<td>1.76</td>
<td>0.78</td>
<td>NA</td>
<td>1.76</td>
<td>2.85</td>
</tr>
<tr>
<td>LOQ, log_{10} IU/mL</td>
<td>3.35</td>
<td>2.14</td>
<td>3.00</td>
<td>2.18</td>
<td>1.94</td>
<td>2.48</td>
<td>1.30</td>
<td>1.49</td>
<td>2.48</td>
<td>2.85</td>
</tr>
</tbody>
</table>

Abbreviations: ASR, assay-specific reagents; CAPCTM, COBAS AmpliPrep/COBAS TagMan CMV Test; CE, Conformité Européenne; CMV, cytomegalovirus; FAM, fluorescent; FDA, US Food and Drug Administration; FRET, fluorescence resonance energy transfer; LD, laboratory developed; LDT, laboratory-developed test; LOD, limit of detection; LOQ, limit of quantitation; MIE, major immediate early; MP, manufacturer provided; NA, not available; PCR, polymerase chain reaction.
Characteristics of CMV PCR Assays

Gene Target:
- DNA polymerase (UL54), glycoprotein B (UL55), UL34/UL80.5, UL83, Major immediate antigen (MIE), others

Amplicon size: 52 to 340 bp
Plasma input volume: 100 to 600 uL
LOD \((\log_{10} \text{IU/mL})\): 0.78 to 3.35
LOQ \((\log_{10} \text{IU/mL})\): 1.30 to 3.35
Different probe chemistry, extraction method, detection method, etc.

Characteristics of CMV PCR Assays

Assay variance:
- Individual CMV DNA-positive samples: median, 1.5 [range, 1.22-2.82] log_{10} IU/mL
- International standard (IS) dilutions: median, 0.94 [range, 0.69-1.35] log_{10} IU/mL (p < 0.001)

58.9% of all clinical sample results and 93.6% of IS dilution results fell within ±0.5 log_{10} IU/mL of the mean

Result variability not impacted by genotype or quantitative levels of CMV DNA

Characteristics of CMV PCR Assays

- For clinical samples all assays demonstrated result bias ($p < 0.008$)
- Assays with amplicon sizes $\leq 86$ bp had significantly higher results compared to those with amplicon sizes $\geq 105$ bp ($p < 0.001$)

Characteristics of CMV PCR Assays

Molecular Assays Licensed by Health Canada - 2017

- Abbott Realtime CMV (Abbott Molecular Inc.)
- Cobas Ampliprep/Cobas Taqman CMV Test (Roche Molecular Systems, Inc.)
- Others available (but not licensed):
  - Luminex
  - Altona
  - Artus
  - In-house assays, others

Abbott RealTime vs Roche CAP/CTM CMV

- 204 plasma samples
- Method bias*: 0.8 log IU/mL
- % Agreement: 83%

*Common method bias refers to a bias in the dataset due to something external to the measures
# Abbott RealTime vs Roche CAP/CTM CMV

<table>
<thead>
<tr>
<th>Abbott RealTime &lt;LOD</th>
<th>Roche CAP/CTM</th>
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</thead>
<tbody>
<tr>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>% Agreement @ LOD</td>
<td>9%</td>
</tr>
<tr>
<td>Roche CAP/CTM &lt;LOD</td>
<td>Abbott RealTime</td>
</tr>
<tr>
<td>41</td>
<td>32</td>
</tr>
<tr>
<td>% Agreement @ LOD</td>
<td>78</td>
</tr>
</tbody>
</table>
Clinical Interpretation of CMV DNA Tests

1. Tests have more variability near the lower LOQ - Assume that changes in viral load must exceed 3-fold (for values in the midrange of the test) to 5-fold (for values in the lower range) to represent meaningful changes in viral replication

2. Factors to consider when interpreting low viral load results include:
   • specimen type, type of transplant, degree of immune suppression, and donor/recipient CMV immune status, LOD and precision of the test

Clinical Interpretation of CMV DNA Tests

3. Monitoring post-transplant: trends in viral load may be more useful than any single viral load value, unless the viral load is very high (>10,000 copies/mL) or the patient is at very high risk (e.g. donor/recipient mismatch).

4. CMV Viral load testing should be performed the day therapy is begun, even if a recent value is available, because viral load levels may increase rapidly in patients with active disease.

5. Follow-up viral load testing should be performed at 5- to 7-day intervals because the half-life of CMV DNA in the plasma ranges from 3 to 8 days. **NB**: CMV DNA may increase in 1st few days after treatment initiated.

Clinical Interpretation of CMV DNA Tests

7. After initiating therapy, viral load should be followed to document clearance of CMV from plasma or whole blood
   - Depending on the initial viral load, this may take weeks or longer

8. Viral load patterns that are worrisome for drug-resistant virus include:
   - those that do not decrease after 2 weeks of adequate therapy
   - those with a plateau in the rate of viral load decline
   - those that have an initial drop and then a subsequent increase in viral load while on therapy

9. Genotypic resistance testing can be performed directly using plasma specimens

Genotypic Testing for CMV Resistance

- No commercially available
- Codons 457 to 630 of the protein kinase (UL97) and codons 393 to 1000 of the polymerase (UL54) CMV genes that harbour known anti-viral resistant mutations that confer resistance to Ganciclovir, Cidofovir and Foscarnet are amplified by PCR and sequenced
- Specimen requirement: CMV positive plasma (0.5 mL minimum), whole blood (1 mL minimum), or viral culture (0.5 mL minimum)
- If CMV viral load <1000 copies/mL may not work
Genotypic Testing for CMV Resistance
Mechanism of Action of Antiviral Drugs for CMV

Mutations Conferring Antiviral Resistance to CMV
Map of the CMV Kinase gene (UL97 or kinase/phosphoptransferase)

*MBV = Maribavir

**UL97 Mutations Conferring Ganciclovir Resistance to CMV** *(Top 7 account for 80%)*

<table>
<thead>
<tr>
<th>Codon number</th>
<th>Wild-type</th>
<th>Mutant</th>
<th>GCV ratio*</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><strong>Canonical mutations</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>460</td>
<td>M</td>
<td>I</td>
<td>5</td>
<td>47, 72, 80, 142, 152-163</td>
</tr>
<tr>
<td>460</td>
<td>M</td>
<td>V</td>
<td>8.3</td>
<td>43, 47, 80, 82, 83, 142, 154-156, 159-161, 164-169</td>
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<tr>
<td>520</td>
<td>H</td>
<td>Q</td>
<td>10</td>
<td>47, 72, 83, 142, 154, 155, 159, 161, 164, 165, 169-171</td>
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<tr>
<td>592</td>
<td>C</td>
<td>G</td>
<td>2.9</td>
<td>72, 80, 82, 83, 142, 153, 154, 159-161, 163, 165, 166, 172, 173</td>
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<tr>
<td>594</td>
<td>A</td>
<td>V</td>
<td>8.3</td>
<td>43, 72, 80, 82, 83, 152-156, 158-161, 164-166, 171, 173</td>
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<tr>
<td>595</td>
<td>L</td>
<td>S</td>
<td>9.2</td>
<td>43, 72, 80, 82, 83, 153-155, 158-166</td>
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<tr>
<td>603</td>
<td>C</td>
<td>W</td>
<td>8</td>
<td>72, 80, 83, 142, 153-155, 159-161, 163, 164, 171, 172, 174, 175</td>
</tr>
<tr>
<td><strong>Other clinically relevant substitution mutations</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>595</td>
<td>L</td>
<td>F</td>
<td>15.7</td>
<td>72, 156, 161, 175</td>
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<tr>
<td>595</td>
<td>L</td>
<td>W</td>
<td>5.1</td>
<td>72, 154, 155, 159-161, 175</td>
</tr>
</tbody>
</table>

*GCV ratio = Mutant IC$_{50}$/Wild-type IC$_{50}$; IC$_{50}$ > 6uM confers resistance

Map of the CMV DNA polymerase gene (UL54 or pol)

## Mutations Conferring Antiviral Resistance to CMV

### Mechanism of Resistance of CMV to Current Therapies

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Analogue Type</th>
<th>Requires UL97</th>
<th>Resistance Due to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ganciclovir</td>
<td>Nucleoside</td>
<td>Yes</td>
<td>UL97 or DNA polymerase mutations</td>
</tr>
<tr>
<td>Foscarnet</td>
<td>Pyrophosphate</td>
<td>No</td>
<td>DNA polymerase mutations</td>
</tr>
<tr>
<td>Cidofovir</td>
<td>Nucleotide</td>
<td>No</td>
<td>DNA polymerase mutations</td>
</tr>
</tbody>
</table>

Boeckh M and Ljungman P. Blood 2009 113
Mutations Conferring Antiviral Resistance to CMV

In 85%–95% of patients, ganciclovir resistance results initially from UL 97 mutations.

If there are verified UL 54 resistance mutations in an isolate obtained from a patient receiving ganciclovir, the virus will be cross-resistant to cidofovir, but cross-resistance to foscarnet is uncommon.

- Mutations of foscarnet resistance will usually only be seen in patients taking foscarnet.

Drew WL. Clin Infect Dis 2010:50
## Mutations Conferring Antiviral Resistance to CMV

<table>
<thead>
<tr>
<th>Site of resistance mutation</th>
<th>Current Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ganciclovir</td>
</tr>
<tr>
<td>UL97</td>
<td>Mediates ganciclovir resistance only</td>
</tr>
<tr>
<td>UL54</td>
<td>Mediates ganciclovir and cidofovir cross-resistance (rarely foscarnet cross-resistance)</td>
</tr>
</tbody>
</table>
Mutations Conferring Antiviral Resistance to CMV

- Takes weeks to months for CMV to develop resistance to antivirals
- Up to two-thirds of patients may exhibit an increase in CMV DNA levels in the first 2–3 weeks of anti-CMV therapy, but this is not due to resistance and should not lead to requests for genotypic assays:
  - The explanation for this phenomenon is unclear, but it appears to be associated with corticosteroid use

Drew WL. Clin Infect Dis 2010:50
Future Testing Options

Molecular (Early/Mid 2000s)

Improved Automation Whole Genome/Next Generation Sequencing (2020 and beyond)
Future Testing Options

Molecular:
- Faster (improved TAT)
- Random access
- High throughput
- Improved standardization
- Lower cost

Resistance testing:
- Deep genome sequencing to detect smaller subpopulations (e.g. currently mutant strains comprising <20 to 30% of the population may not be detected/reported; Whole genome sequencing?)
- Commercialized, standardized assays
- New drugs with new mechanisms of action will require testing for drug resistance and correlation between genotype and phenotype
“Happy Herpes”
Thank you for your attention!