Detection of (drug-resistant) cytomegalovirus in immunosuppressed patients

An overview

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HCMV - diagnostics

- **Serology**
  - IgG, IgM, (avidity)
  - 6 h to 24 h

- **Antigenemia**
  - (pp65)
  - 5 to 6 h

- **Real-time PCR**
  - DNA
  - 2 to 4 h

- **Quantiferon-CMV ELISA**
  - (interferon-γ)
  - 24 h

- **Shell vial virämia**
  - 1 to 3 days

- **Virus culture isolate**
  - 1 to 6 weeks

- **Phenotyping**
  - (after virus isolation 1-2 weeks)
  - (ganciclovir, foscarnet, cidofovir)

- **Genotyping**
  - UL97
  - 2-3 days
  - Polymerase (UL54)
  - 4-7 days
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HCMV-serology and transplantation

- serology should be performed before transplantation on both donor and recipient

- donor and recipient serostatus (D/R) are key predictors of infection risk and management

- no role in the diagnosis of active HCMV disease after transplantation

- interpretation of results can be difficult:
  - in D/R with recent transfusion of blood products
  - in children younger than 12 months, as passive transfer of antibody can lead to transient false-positive serologic results
HCMV - diagnostics

- **Genotyping**
  - UL97: 2-3 days
  - Polymerase (UL54): 4-7 days

- **Phenotyping**
  - After virus isolation (1-2 weeks)
  - (ganciclovir, foscarnet, cidofovir)

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- **Other**
  - Virus culture isolate (after virus isolation)
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- Shell vial virämia 1 to 3 days
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Genotyping
- UL97 2-3 days
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HCMV monitoring after transplantation

- qPCR is preferred for diagnosis, decisions regarding preemptive therapy, and monitoring response to therapy
  - due to the ability to harmonize and standardize these tests

- commercial and in-house tests must be calibrated to the WHO international standard
  - results have to be reported as IU/ml

- either plasma or whole blood is an acceptable specimen for qPCR
  - specimen type should not be changed when monitoring patients

- if qPCR is not available, antigenemia is an acceptable alternative
pp65 antigenemia

• does not require expensive equipment and is relatively easy to perform

• lack of standardization, including subjective result interpretation
  – it is unlikely that better standardization of this assay will occur, because most laboratories have moved to molecular methods

• assay performance diminishes when the absolute neutrophil count is less than 1000/mm³

• blood specimens should be processed as fast as possible to avoid a decrease in test sensitivity
  – thus, transplant centers managing patients at distant sites whose blood samples are mailed into the laboratory may prefer to use qPCR rather than antigenemia
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**Diagnosis**
- Real-time PCR
  - 2 to 4 h
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Mechanism of action of anti-cytomegalovirus (HCMV) drugs

Valganciclovir (ValGCV) is hydrolyzed to ganciclovir (GCV) in mucosa cells. GCV is transported into infected cells and phosphorylated by CDV kinases to a monophosphate (P), which is further phosphorylated by cellular kinases to a triphosphate (P3). The viral DNA polymerase (UL97) is then inhibited by phosphorylated ganciclovir (P3), leading to the inhibition of viral DNA synthesis. Foscarnet (FOS) inhibits viral DNA polymerase directly, while cidofovir (CDV) inhibits the viral DNA polymerase indirectly by acting on viral DNA.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Probability</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-infection (AIDS)</td>
<td>2%</td>
<td>&gt; 3 months</td>
</tr>
<tr>
<td>transplantation, general</td>
<td>9%</td>
<td>9 months</td>
</tr>
<tr>
<td>lung transplantation, D+/R-</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td>kidney-/liver transplantation</td>
<td>&lt;10%</td>
<td></td>
</tr>
<tr>
<td>lung transplantation, D+/R-</td>
<td>30%</td>
<td>(&gt;2 months)</td>
</tr>
</tbody>
</table>
Probability of viral resistance of HCMV
In immunosuppressed patients

Long term therapy:

<table>
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<tr>
<th>Procedure</th>
<th>Probability</th>
<th>Time</th>
</tr>
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<td></td>
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</table>

Our results after suspicion of viral resistance: (n ~ 1500 patients):

- 15%    PCR/pp65 negative
- 50%    wild-type
- 35%    GCV/FOS/CDV resistance
Impact of different mutations in UL97 on drug sensitivity

* >85% using Genotypic resistance assay
From genotype to phenotype to consequences for therapy

Adherence to ganciclovir therapy

- A478V
- T601M
- V466M
- C603del
- A497T
- D605E
- L600I
- N597D
- H469Y
- K599E
- A591V
- C603S
- C592G
- A594E
- C607F
- E596G
- G598S
- M460V
- L592S
- A594V
- H520Q

Switch to FOS is suggested

Maintain therapy may permit GCV at higher doses
Distribution of UL97 mutations detected by genotyping (n = 235)
This service is new! Although we simulated several test data, we cannot guarantee for perfect performance at the moment. In case of any problems or suggestions do not hesitate to contact us (hans.kestler [at] uni-ulm.de).

**Mutation Analyzer**

Optional personal identifier: 

Do not use name of patients!

Submit a sequence and choose the gene you want to align to (sequences in FASTA format are accepted also.):

Upload a file: 

Select the gene you want to align to:
- HCV (NS3) (Detail)
- HSV-1 (UL23/TK) [under development] (Detail)
- HSV-1 (UL30) [under development] (Detail)
- CMV (UL54) (Detail)
- CMV (UL97) (Detail)

I have read the terms of use and agree with them: 

I want to use the worst case calculation for the wild bases: 

The calculation will take some time, depending on the length and the quality of the sequence (~ 20-60 seconds). Sequences containing wild bases will take more time!

Daten absenden
### Data for UL54-test-sequence (UL54-test-sequence) compared with UL54

#### Mutations

<table>
<thead>
<tr>
<th>S/N</th>
<th>Position</th>
<th>Wild Type AA</th>
<th>Submitted AA</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>413</td>
<td>D</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>898</td>
<td>D</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>1122</td>
<td>T</td>
<td>A</td>
</tr>
</tbody>
</table>

#### Map of Mutations

- alignment
- mutation not in database
- mutation with unclear phenotype
- mutation associated with drug resistance
- mutation associated with drug susceptibility

#### List of all found mutations: D413A, D898N, T1122A

#### Information

<table>
<thead>
<tr>
<th>S/N*</th>
<th>locus</th>
<th>mutation</th>
<th>resistant**</th>
<th>sensitive**</th>
<th>GCV***</th>
<th>CDV***</th>
<th>FOS***</th>
<th>viral fitness</th>
<th>literature</th>
<th>method of testing</th>
<th>additional Information</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>898</td>
<td>D898N</td>
<td></td>
<td></td>
<td>GCV, CDV, FOS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>sequence alignments of sensitive laboratory strains</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>413</td>
<td>D413A</td>
<td>GCY, CDV</td>
<td>FOS</td>
<td>6.5x</td>
<td>10.9x</td>
<td>0.8x</td>
<td>slightly impaired</td>
<td>Marfort et al., 2007</td>
<td>marker transfer (not clonal); SEAP assay</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1122</td>
<td>T1122A</td>
<td>GCY, CDV</td>
<td>FOS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chou et al., 1999</td>
<td>clinical isolate; plaque reduction assay</td>
<td></td>
</tr>
</tbody>
</table>

* sorting: “category of test” ascending
** as stated by the respective authors
*** drug susceptibility (ratio of EC50)

Folders will be deleted after 7 days.

# Requests to the MRA-website (UL97 / UL54)

[ww.informatik.uni-ulm.de/ni/mitarbeiter/HKestler/mra/app](http://www.informatik.uni-ulm.de/ni/mitarbeiter/HKestler/mra/app)

<table>
<thead>
<tr>
<th>year</th>
<th>MRA-HCMV</th>
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<tbody>
<tr>
<td>2010</td>
<td>2019</td>
</tr>
<tr>
<td>2011</td>
<td>2798</td>
</tr>
<tr>
<td>2012</td>
<td>4911</td>
</tr>
<tr>
<td>2013</td>
<td>5555</td>
</tr>
<tr>
<td>2014</td>
<td>5447</td>
</tr>
<tr>
<td>2015</td>
<td>6976</td>
</tr>
<tr>
<td>12.02.2016</td>
<td>661</td>
</tr>
</tbody>
</table>
„Evolution“ of resistant HCMV

UL97
L595S 20%
H411Y 20%
D605E
Pol
T700A 20%
A809V 20%

UL97
L595S 20%
D605E
Pol
wt

UL97
M460V 20%
L595S 50%
D605E

UL97
wt
D605E
Pol
wt

UL97
T409A 50%
H411Y 50%
D605E
Pol
n.d.

foscarvir
ganciclovir
cidofovir
maribavir
leflunomid
Cloning proofs accumulation of different Maribavir-resistant strains under therapy

direct sequencing from EDTA-blood

Cloning in E. coli

clone 1

clone 2

clone 3
Observations during anti-HCMV therapy

- mutations are distributed among different subpopulations (strains) of the same clinical isolate

- several strains/variants may "disappear" under therapy (i.e. M460I), others maintain

- during ganciclovir therapy pauses (under foscarin or without) "repopulation" with wildtype can occur

- GCV-resistant variants have a selection advantage if therapy is continued

Quellen:
Schubert et al. 2013 BMC Infect Dis
Drew and Liu 2012, Clin Transplant
Strasfeld et al. 2010, J Infect Dis
Genotyping is expensive (?)
Daily costs of anti-HCMV therapy
(according prime costs)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cost</th>
<th>Dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ganciclovir (i.v.)</td>
<td>68,00 €</td>
<td>(2x5mg/kg)</td>
</tr>
<tr>
<td>Valganciclovir (p.o.)</td>
<td>101,00 €</td>
<td>(2x900 mg)</td>
</tr>
<tr>
<td>Foscarnet</td>
<td>382,00 €</td>
<td>(2x90 mg/kg)</td>
</tr>
<tr>
<td>Cidofovir</td>
<td>67,00 €</td>
<td>(1x5 mg/kg/2 weeks; one vial 961,00 €)</td>
</tr>
<tr>
<td>(Artesunate)</td>
<td>(68,00 €)</td>
<td>(2,4 mg/kg)</td>
</tr>
<tr>
<td>(Leflunomide)</td>
<td>(3,00 €)</td>
<td>(20 mg )</td>
</tr>
</tbody>
</table>
Molecular aims of the HCMV therapy

- Ganciclovir
- Cyclopropavir
- Maribavir
- UL97 (Replication enzyme)
- UL54 (DNA Polymerase)
- UL56

- Egress of the linear double-strand genome
- Packaging: Import of DNA into capsid. Cutting to genome lengths
- Complex of UL56/UL89 proteins with capsid/DNA
- UL89

- Envelopment and egress
- Letermovir
- Artesunate?
- Leflunomid?

- Foscarnet
- Cidofovir
- Maribavir
- Cyclopropavir
- Brincidofovir
Summary: HCMV and resistance

- different diagnostic tools for the diagnosis of active HCMV infections are available
- UL97 and UL54 genotyping is suitable for adjustment of therapy
- different mutations in the same “patient virus population” are mostly distributed among different virus strains
- in patients consecutively treated with different compounds “virus evolution” can be observed
- new mutations require a continuously updated public database of the impact on the viral phenotype
- new substances may require new genotypic (phenotypic?) assays (Letermovir, Maribavir, Artesunate, Leflunomid)
Ulm definition of phenotypic GCV resistance

number of tested HCMV isolates

90% perzentil

median 3.6 µM

sensitive

reduced sensitive

resistent

Ganciclovir HD$_{50}$ (in µM)
When do we have to start therapy?

- few studies define trigger points for intervention therapy when using a preemptive approach

- higher viral load values correlate with increased risk for disease

- one study established a cutoff for predicting disease of 2000 to 5000 copies/ml in plasma in HCMV seropositive liver transplant recipients, using qNAT
  - this cutoff may not apply to different specimen types, in different populations and risk groups

- one study of low-risk HCMV seropositive kidney, heart, and liver transplant recipients not receiving antilymphocyte globulins suggested a preemptive therapy trigger point of 3893 IU/ml plasma

- viral load kinetics (rapid doubling time) in high-risk groups suggests that the frequency of viral load testing will impact the effectiveness of a preemptive strategy (i.e. more frequent testing will be more effective)

Viral culture of blood for HCMV has limited clinical utility for diagnosis of disease due to poor sensitivity.

There is no role for HCMV urine culture in the diagnosis of disease due to poor specificity.

Viral load testing is the cornerstone for diagnosis and monitoring for CMV infection and disease; both qNAT and antigenemia testing are available for these purposes.

The HCMV pp65 antigenemia test is a semiquantitative test that is useful for the diagnosis of clinical disease, initiating preemptive therapy and monitoring response to therapy.

Studies have shown that higher numbers of positive staining cells correlate better with disease although tissue-invasive disease can occur with low or negative cell counts.
Pyrosequencing/deep sequencing/whole genome sequencing für die Resistenztestung

• Anteile von 1-5% an Varianten in einem Isolat (?)/Amplikon können identifiziert werden

• Bewertung verschiedener antiviraler Targets simultan möglich

• “Evolution” der im Patienten unter Therapie replizierenden Varianten zeitnah nachvollziehbar

• Frage der Konsequenzen noch unklar
Mutations in the viral polymerase
Ratio of IC$_{50}$ strain/WT

\[
\text{Ratio} = \frac{\text{IC}_{50} \text{ mutant}}{\text{IC}_{50} \text{ wild type}}
\]

1 ≤ sensitiv/potential resistance ≥ 2 ≤ potential/low resistance ≥ 3 ≤ low to high resistance
<table>
<thead>
<tr>
<th>Subheading</th>
<th>Description</th>
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<tbody>
<tr>
<td>„Alternativen“ zur bisherige anti-HCMV-Therapie</td>
<td></td>
</tr>
<tr>
<td>Leflunomide</td>
<td>wirkt scheinbar langsam (eventuell Wochen bis Viruslast signifikant sinkt)</td>
</tr>
<tr>
<td>Artesunate</td>
<td>divergente Ergebnisse in klinischen Studien Teilweise Ineffektivität gegenüber resistenten Varianten</td>
</tr>
<tr>
<td>Cyclopropavir</td>
<td>muss durch UL97 initial monophosphoryliert werden Kreuzresistenz mit GCV</td>
</tr>
<tr>
<td>CMX-001</td>
<td>oral, Lipidester von Cidofovir; gleicher Mechanismus! geringere Toxizität?</td>
</tr>
<tr>
<td>Letermovir</td>
<td>oral, interagiert mit viraler UL56 Untereinheit erfolgreich bei “multidrug”-resistenten Varianten</td>
</tr>
<tr>
<td>Adoptiver T-Zell Transfer</td>
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