TransMedRI

# Workshop on Congenital Infections

*Rijeka, May 21st – 22nd, 2012*
*University of Rijeka, Faculty of Medicine*
*Department of Histology & Embryology*

*Moderated by*
*Ulrich Koszinowski and Stipan Jonjić*

TransMedRI

# Workshop Program

**Day I - May 21st Lecture Hall**

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|---|---|
| 09:00 - 09:45 | **William Britt**<br>Maternal immunity and congenital CMV infection |
| 09:45 - 10:30 | **Arnaud Marchant**<br>Fetal immunity to cytomegalovirus |
| 10:30 - 11:15 | **Tiziana Lazzarotto**<br>Diagnosis of infection in adults (pregnant women) fetuses and newborns |
| 11:15 - 11:45 | *Coffee break* |
| 11:45 - 12:30 | **Suresh Boppana**<br>Newborn CMV Screening |
| 12:30 - 13:15 | **Milena Furione**<br>Other viral (rubella, parvoB19 and VZV) congenital infections |
| 13:15 - 14:00 | *Lunch* |
| 14.00 - -- | Joint Steering Committee & Advisory Board Meeting<br>TransMedRi project |
| 14:30 – 18.00 | ***Practical part****<br>- Determination of cytomegalovirus (CMV) IgG in sera<br>- High throughput DNA extraction from blood using MagNA Pure LC 2.0 robot |

**Day II - May 22nd Lecture Hall**

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|---|---|
| 09:00 - 09:45 | **Dana Wolf**<br>Better understanding of congenital CMV: from modeling of maternal-fetal transmission to neonatal screening |
| 09:45 - 10:30 | **Vlatka Mejaški Bošnjak**<br>Malformations of Cortical Development in Children with Congenital CMV Infection |
| 10:30 - 11:15 | **Maria Grazia Revello**<br>Clinical trials for prevention of congenital CMV infection |
| 11:15 - 11:45 | *Coffee break* |
| 11:45 - 12:30 | **Goran Tešović**<br>Congenital CMV-infection: The Croatian Experience |
| 12:30 - 13:15 | **Peter Huszthy** and **Jurica Arapović**<br>MCMV model of congenital CMV infection-progress report |
| 13:15 - 14:30 | *Lunch* |
| 14:30 - 15:30 | ***Round table*** |
| 15:30 – 18.00 | ***Practical part****<br>- Detection of CMV in blood by qPCR |

* *all practical parts of the workshop to take place at the Center of Proteomics*

**Day I: practical part:**

- **DETERMINATION OF CYTOMEGALOVIRUS (CMV) IgG IN SERA**

**Method ELISA: Enzyme Linked Immunosorbent Assay**
*ELISA CMV IgG is an accurate serologic method to detect CMV antibody for identification of CMV infection.*
**Principle ELISA – Indirect, Antigen Coated Plate**

*background info*
*About half of pregnant women who contract a primary infection spread the disease to their fetus. Serological tests for detecting the presence of antibody to CMV can provide valuable information regarding the history of previous infection, diagnosis of active or recent infection, as well as inscreening blood for transfusions in newborns and immuno-compromised recipients.*

*Method principles:*
*Purified CMV antigen is coated on the surface of micro-wells. Diluted patient serum is added to wells, and the CMV IgG specific antibody, if present, binds to the antigen. All unbound materials are washed away. After adding enzyme conjugate, it binds to the antibody-antigen complex. Excess enzyme conjugate is washed off and TMB Chromogenic substrate is added. The enzyme conjugate catalytic reaction is stopped at a specific time. The intensity of the color generated is proportional to the amount of IgG specific antibody in the sample. The results are read by a micro-well reader compared in a parallel manner with calibrator and controls.*

**MATERIALS PROVIDED**
Kit: The DIAGNOSTIC AUTOMATION ELISA, CMV IgG/
intended for use in evaluating serologic status to CMV infection.

**SPECIMEN COLLECTION AND HANDLING**
1. Collect blood specimens and separate the serum.
2. Specimens may be refrigerated at 2 - 8$^{o}$C for up to seven days or frozen for up to six months. Avoid repetitive freezing and thawing of serum sample.

**PREPARATION FOR ASSAY**
1. Prepare 1x washing buffer.
   (*Prepare washing buffer by adding distilled or deionized water to 10x wash concentrate to a final volume of 1 liter*)
2. Bring all specimens and kit reagents to room temperature (20-25$^{o}$C) and gently mix.

**ASSAY PROCEDURE**
1. Place the desired number of coated strips into the holder.
2. Prepare 1:40 dilutions by adding 5 µl of the test samples, negative control, positive control, and calibrators to 200 µl of sample diluent. Mix well.
3. Dispense 100 µl of diluted sera, calibrator, and controls into the appropriate wells. For the reagent blank, dispense 100 µl sample diluent in 1A well position. Tap the holder to remove air bubbles from the liquid and mix well. Incubate for 30 minutes at room temperature (RT).
4. Remove liquid from all wells. Repeat washing three times with washing buffer.
5. Dispense 100 µl of enzyme conjugate to each well and incubate for 30 minutes at RT.

6. Remove enzyme conjugate from all wells. Repeat washing three times with washing buffer.
7. Dispense 100 µl of TMB Chromogenic Substrate to each well and incubate for 30 minutes RT in a dark place or cover the plate.
8. Add 100 µl of 2 N HCl to stop reaction. Make sure there are no air bubbles in each well before reading.
9. Read O.D. at 450 nm with a microwell reader.

**CALCULATION OF RESULTS**

1. Calculate the mean of duplicate calibrator value.
2. Calculate the mean of duplicate positive control, negative control and patient samples.
3. Calculate the CMV G Index of each determination by dividing the mean values of each sample by calibrator mean value.

**Example of typical results:**

Calibrator (1.2 IU/ml); mean value O.D = 1.215
Patient sample; mean value O.D. = 2.309
**CMV G Index** = 2.309 / 1.215 = 1.9

| Cut-off Calibrator CMV G Index = 1.0 |
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**INTERPRETATION OF RESULTS:**

**Negative**: CMV G Index of 0.90 or less are seronegative for IgG antibody to CMV. ( <1.1 IU/ml )
**Equivocal**: CMV G Index of 0.91-0.99 are equivocal. Sample should be retested.
**Positive**: CMV G Index of 1.00 or greater, or IU value greater than 1.2 are seropositive. It indicates prior exposure to the CMV virus. ( > 1.2 IU/ml )

**Day I: practical part:**

- **High throughput DNA extraction from blood using MagNA Pure LC 2.0 robot**

*MagNA Pure LC 2.0 Instrument robot – general instrument capacity:*

1. *automated isolation of nucleic acids (DNA, total RNA, total viral nucleic acids) from different kinds of biological research sample material (whole blood, serum, blood cells, culture cells, tissue, bacteria, fungi, etc.)*
2. *specially designed MagNA Pure LC Kits*
3. *up to 32 (4 x 8) samples can be processed in one run*
4. *Sample volume 20 to 1000 µl*
5. *Elution volume 50 to 200 µl*
6. *Processing time Approx. 60 to 180 min, depending on the protocol type and sample number*

*Material that will be used within the workshop::*

1. Starting material: core blood
2. Kit choice: *MagNA Pure LC Total Nucleic Acid Isolation Kit – High Performance*
3. total sample number: 2*32 samples
4. each sample volume: 100 ul
5. elution volume: 50ul, PBS(*Phophate buffered saline*)
6. processing time (one round): 90 min

*Procedure:*

1. start the MagNA Pure LC 2.0 Software (user: Admin; pass:Admin0)
2. choose the purification protocoland parameters:
    - workplace menu (activate 1 sample first);
    - protocol: total NA
    - sub tab: total NA; variabile vol:
        - Sample: 100ul
        - Eluion: 50ul
    - Activate all 32 samples (add names);
      *Parameters defined for the first sample will be automaticaly applied*
    - SAVE/LOAD

3. Type and amount of isolation reagents and disposable plastics required for the purification run are automatically calculated by the software, according to input parameters.
    - this information is displayed on the *Stage Setup* sub-tab.

4. Place disposable Plastics in appropriate holders

5. Pipet isolation reagents into nuclease-free, disposable Reagent Tubs

6. The instrument automatically performs all remaining steps of the procedure using specially designed nuclease-free, disposable Reaction Tips.
   *Reaction Tips not only transfer the samples, but also serve as reaction vials where nucleic acids are bound to magnetic beads, washed free of impurities and finally eluted from the magnetic beads.*

7. Store nucleic acids, eluted in 50ul volume, from the Storage Cartridge for further use.
   *After completion of a nucleic acid purification run, the MagNA Pure LC 2.0 Instrument can be instructed to transfer the eluted nucleic acid samples into LightCycler® Capillaries,wells of a PCR plate, or reaction tubes. Also, the set-up of PCR master mixes or dilution series can be performed automatically by the Instrument.*

**Day II: practical part:**

- **Detection of CMV genome in blood by qPCR**

Based on Boppana *et al*. (2010) JAMA
**Materials**:

1. primers for amplification of HCMV gB gene:
    a. gB forw: 5'- AGG TCT TCA AGG AAC TCA GCA AGA
    a. gB rev: 5'-CGG CAA TCG GTT TGT TGT AAA
2. plasmid containing gB gene for titration curve

**Primers**: concentration of main stock: 100 umol/ml
working stock – 5-10 umol/mL. You can make a premixed primer stocks with both primers.

**Titration curve for determination of CMV genome copies**
pCR2.1 plasmid containing gB , size: 4kb
1bp= 660 Da = 660 g/mol
1 molecule of plasmid =660 g/mol * 4000 bp = $2{,}64*10^6$ g/mol
Make 5 decimal dilutions of plasmid: (npr. 100 000, 10 000, 1000, 100, 10 plasmid copy/ul)

Everything is done in triplicates or duplicates.
No template control: 2 samples containing everything but DNA.

**Typical qPCR reakcija on ABI 7500 Fast (max volume of reaction is 20 ul)**
5 ul DNA
10 ul SYBRGreen mastermix
5 ul primer mix (c of each primer in mix is 5 umol/ul)

**example of plate set-up:**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | PCR2.1 $10^5$ | PCR2.1 $10^4$ | PCR2.1 $10^3$ | PCR2.1 $10^2$ | PCR2.1 10 | | | | | | | |
| B | PCR2.1 $10^5$ | PCR2.1 $10^4$ | PCR2.1 $10^3$ | PCR2.1 $10^2$ | PCR2.1 10 | | | | | | | |
| C | PCR2.1 $10^5$ | PCR2.1 $10^4$ | PCR2.1 $10^3$ | PCR2.1 $10^2$ | PCR2.1 10 | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | Sample 1 | Sample 2 | | No template control | | | | | | | | |
| G | Sample 1 | Sample 2 | | No template control | | | | | | | | |
| H | Sample 1 | Sample 2 | | No template control | | | | | | | | |

**Notes:**