Assays for the detection of human cytomegalovirus infection

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In tissue and organ transplantation the imunosuppresive therapy produces an environment in which human cytomegalovirus (HCMV) can exert its full pathogenicity. In the immunocompromised host HCMV disease is a serious cause of morbidity and mortality. Early treatment of HCMV disease, or preemptive therapy is mandatory for the patient and the graft. Organ transplant centers must be supported by laboratories capable to trace routinely the replication of HCMV. The methods used today are described in this technical note and include non-molecular assays (pp 65 antigenemia assay) and molecular assays (CMV - DNA quantitative PCR assay). *Hippokratia 2003, 7 (2):93-96*

The HCMV is the largest in size among the few human herpes viruses (the others are: HSV-1, HSV-2, VZV, EBV, HHV-6, HHV-7). These viruses are members of the Herpesviridae family and are double-stranded DNA viruses in the form of an icosahedron.

The virus particle consists of the core DNA enclosed by protein capsomeres and the unit which is embedded in a loosely-structured matrix (the tegument) and a membrane (envelope) containing virus specific glycoproteins, which is acquired during budding through host cellular membranes. This constitutes the mature infectious virus with a variable size of 150-300 nm.

HCMV attaches to specific receptor on cell and enters by fusion of envelope with cell membrane in order to replicate. The release of the virus DNA (the uncoating) takes place at the nuclear pores; then it enters the cell nucleus. At this time point the viral metabolism starts and proceeds rather sluggishly in the case of HCMV and requires long surviving intact cell functions. As with all other herpes viruses the HCMV synthesis of various proteins essential for its replication proceeds in a cascade of three stages. First CMV-DNA genes are transcribed for the synthesis of the α-proteins i.e. the immediate early antigens. These stimulate synthesis of the β-proteins (early antigens) which, too, are regulator proteins or enzymes, including the DNA polymerase. Only during the third cascading stage are the γ-proteins (late antigens) generated which, as structural proteins, together with the newly synthesized DNA, serve to develop new virus particles. The assembly of nucleocapsids takes place in the cell nucleus and then they bud through nuclear membrane, but lose this membrane in the cytoplasm in order to develop a final virus membrane by budding through membranes of Golgi's apparatus. With HCMV relatively little virus is released; most of the infectious particles remain cell-bound. The monocytes/ macrophages serve as target cells for latent persistent infection, while the salivary glands and the renal tubular epithelia, along with the mucus membranes of the genital tract are the primary sources for discontinuous virus secretion[1].

Diagnostic Methods

A. Non-Molecular Assays

I. Conventional Cell Culture

The specimen is inoculated onto human embryo fibroblast monolayers, only where CMV can multiply. The infected cells round up and fuse with adjacent cells to form syncytia. This cytopathic effect (CPE) is visible as a localized plaque in 1 to 4 weeks, depending on the quantity of the virus in the sample. Confirmation requires the use of CMV-specific monoclonal antibodies (Abs).

Due to its low sensitivity (very slow CPE), nowadays its value is limited to antiviral drug susceptibility testing[2].

II. Shell Vial Assay

It has replaced the previous method, as it is much quicker in yielding results. In this test the inoculation of various clinical specimens is aided by their

centrifugation onto cover slips with a culture cell monolayer to increase the rapidity and efficiency of cell infection.

After 2-3 days the infected cells have developed an "immediate early antigen" (p72), which can be detected by staining with the corresponding monoclonal Abs and visually scored as a number of fluorescent foci.

With a constant viral inoculum the assay can be used quantitatively, but due to its low sensitivity it is not suitable for preemptive therapy[3].

III. pp65 Antigenemia Assay

This immunocytochemical technique detects the HCMV virus structural protein pp65 (or immediate early antigen) in the patient's PMNs. The latter must be isolated from blood and centrifuged onto at least two glass slides within 4 hours to a concentration of 2×10^5 leukocytes per slide. After fixation the infected cells are directly stained with CMV pp65 monoclonal Abs and examined microscopically. The results are expressed as the number of pp65 – positive cells relative to the number of cells used to prepare the slide.

It is a rapid (5-6 hours), sensitive method with a high positive predictive value and thus is recommended for preemptive therapy and response to treatment (at a threshold of $= 10$ positive cells/ 2×10^5 leukocytes for solid organ transplantation)[4]. It does not require a highly specialized laboratory.

Disadvantages are the rather low reproducibility, the need for fast processing, the time consuming nature of the assay steps and the subjective interpretation of the results. In addition it is not suitable if the patient has neutropenia. Misinterpretation may arise with low levels of pp65 antigenemia positive cells which can be present during abortive/transient infections. Since this protein is an early gene product, it can be expressed during these stages[5]. The presence of pp65 antigen in circulating leukocytes does not reflect local protein production and is probably due to phagocytosis rather than de novo synthesis[6].

B. MOLECULAR ASSAYS

I. Hybrid Capture CMV DNA Assay

Patient's whole blood containing the target DNA is denatured and hybridized with a specific HCMV RNA probe which is complementary to about 17% of the HCMV genome. The resulting DNA-RNA hybrids are captured in a tube with adsorbed hybrid-specific Abs. Then alkaline phosphatase-conjugated Abs specific for the hybrids are added, which hydrolyze a chemiluminescent substrate. A DNA-RNA duplex binds about 1000 antibody conjugates, each of which has 3 alkaline phosphatase molecules. Thus the signal is amplified at least 3000 times. The amount of light emitted is analogous to the amount of target DNA in the sample. (turnaround time about 6 hours).

This method is both quantitative and qualitative with high sensitivity, suitable for guiding preemptive treatment and monitoring therapy efficacy[7,8].

II. Branched-DNA (bDNA) Assay

It is based on signal amplification with bDNA multimers which provide multiple binding sites for an enzyme-labeled probe. The bDNA probe is detected with a chemiluminescent substrate in which the light output is directly proportional to the amount of DNA in the original sample (at least 2×10^6 leukocytes needed). After release and denaturation of the CMV DNA, it is hybridized to specific target probes and to microwells through capture probes. Then synthetic bDNA amplifier molecules are hybridized to immobilized target hybrids in the microwells. Signal amplification is achieved through the multiple repeat sequences within each bDNA multimer that serve as sites for hybridization with alkaline phosphatase-conjugated oligonucleotide probes. The chemiluminescent substrate is added and the light emission measured[9].

The 2nd generation assay appears to be as sensitive as the pp65 antigenemia test and has high reproducibility, but requires a large number of PMNs and has long initial incubation period (16-18 hours) [2].

III. NASBA CMV pp67 Assay

This assay detects mRNA coding for the pp67 (UL65) of HCMV, a true late protein that is only expressed during viral replication. Based on the Nucleic Acid Sequence-Based Amplification (NASBA) technology it selectively amplifies RNA in a DNA background and allows direct testing of whole blood. After using a lysis buffer to inactivate viral activity in the sample and to stabilize the released nucleic acids, the latter are then purified through binding to silica particles. The late HCMV mRNA is amplified with the NASBA or TMA (Transcription-Mediated Amplification) isothermal technique, which relies on the simultaneous activity of 3 enzymes (AMV-reverse transcriptase, RNase H and T7 RNA polymerase) and two primers (one with T_7 promoter). Each of the newly synthesized RNA amplicons reenters the TMA process and serves as a template for a new round of replication leading to an exponential expansion of the tar-

get RNA. The RNA amplicons are indirectly detected by a one-step probe hybridization assay using electro-chemiluminescence. An internal control of similar CMV RNA is added to each sample prior to extraction, isolated and amplified in the same tube and separately detected. The amount of CMV RNA is determined from the ratio of the signal of the sample RNA divided by the signal of the control (turnaround time about 6 hours).

As the pp67 is only expressed during viral replication, the assay measures active infections only, unlike PCR, which can be too sensitive and detects both latent and active disease states[2]. The HCMV pp67 provides earliest and true indication of therapeutic efficacy. CMV mRNA disappears quickly, weeks before pp65 antigenemia and DNA copies begin to decline[10]. The expression of pp67 RNA is more sensitive than the pp65 antigenemia test in renal transplant patients. Both are very specific and highly predictive of the onset of HCMV infection[11]. The CMV pp67 assay is also helpful in tracing emerging resistance and /or slow responders. While pp65 antigenemia decreased and therapy therefore was stopped, the HCMV pp67 remained positive, indicating continued replication of the virus[12].

Any positive value for pp67 was indicative for active infection and quantification of it did not give additional diagnostic information[13] .

In conclusion, the NASBA CMV pp67 appears to be a sensitive, specific, promising and well-standardized tool for determination of the need for initiation and termination of preemptive therapy[5] .

IV. CMV- DNA Qualitative PCR Assay

The polymerase chain reaction (Chemistry Nobel 1993) can selectively and repeatedly replicate selected segments from a complex DNA mixture. This way of amplifying rare known sequences from a mixture has vastly increased the sensitivity of genetic tests.

In a typical application of PCR, DNA from a small sample of blood is denatured into single strands at 95°. Oligonucleotide probes (primers) complementary to the 3' ends of the DNA segment to be amplified are added at 50-60^0 C; these hybridize with the correct site on the DNA. The hybridized probes will then serve as primers for DNA chain synthesis, which begins upon addition of a supply of deoxynucleotides and a temperature-resistant DNA polymerase (obtained from Thermus aquaticus and able to extend the primers at 72^{0}C). Then the whole mixture is heated further to 95^{0}C to melt the newly formed DNA duplexes. When the

temperature is lowered again, another round of synthesis can take place because excess primers and nucleotides are still present. This cycle can be repeated and at each round the copies of the desired DNA sequence increase exponentially [14].

After extraction of the CMV-DNA, both the target DNA and the internal control (included with each sample to check for the presence of PCR inhibitors) are amplified using biotinylated primers for the CMV-DNA polymerase gene. The amplified products are then hybridized to specific probes in a microwell plate and detected by addition of avidin-peroxidase conjugate and substrate (turnaround time about 6 hours).

It is a fast and more sensitive method than the detection of viremia or antigenemia. However, PCR may detect latent virus in healthy CMV sero- positive individuals and has a low positive predictive value for CMV disease in the transplant setting [15] .

V. CMV-DNA Quantitative PCR Assay

It is a very sensitive assay, which utilizes a complex system where amplification, denaturation, hybridization, incubation, washing, reading and result reporting are all performed automatically in less than 6 hours. On the other hand, it is an expensive method which does not properly estimate the viral load when concentrations greater than 10^5 copies per ml are present in the sample[16]. Its sensitivity is higher on leukocyte assays than assays on plasma, but plasma PCR is easier to perform (easier specimen processing), it is possible to detect CMV in cases of neutropenia, and it gives fewer sporadic, non-consecutive, positive results, which need to be repeated[17].

IV. Other Molecular Methods

Molecular assays are now considered to be the «gold standard» for assesment of HCMV infection and disease in those at risk from severe associated clinical manifestation.

Except for the previously mentioned and the in house-assays, other molecular techniques have been recently performed, like reverse transcriptase PCR, Real-Time PCR, NASBA for the detection of other HCMV genes [as the UL123=IE1, US3, US6, US11], Multiplex Real-Time NASBA for the simultaneous detection of immediate early 1 and late pp67 mRNA [18].

The IE1 NASBA assay had higher sensitivity than the NASBA pp67 and even than the HCMV-DNA PCR, being an attractive screening test for the early detection of HCMV infection and the initiation of preemptive therapy in transplant

recipients[19,20].

More large, comparative clinical studies using different molecular standardized strategies must still be carried out to determine their respective diagnostic values.

C. ASSAYS BASED ON SEROLOGY AND HISTOPATHOLOGY

These are no longer used for guiding preemptive therapy. Serological diagnosis gives only indirect evidence of the presence of the virus and can be problematic, due to the immunological disorders occurring in most patients at risk of developing HCMV infection[21]. Neither seroconversion nor determination of the presence of IgM Abs reliably reflects viral replication[16].

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