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Evaluating Cytomegalovirus (CMV): Laboratory Diagnostic Assays: Standardization, Sensitivity, and Algorithm Design. A Literature Review.

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Abstract: Cytomegalovirus (CMV) is a globally distributed herpesvirus that establishes lifelong latency and can cause severe disease in newborns, pregnant women and immunocompromised patients. Accurate laboratory diagnosis is essential to guide antiviral therapy, infection control, and epidemiological surveillance. Multiple diagnostic modalities are used, including nucleic acid amplification testing (NAAT) such as polymerase chain reaction (PCR) and transcription-mediated amplification (TMA), clustered regularly interspaced short palindromic repeat (CRISPR-based assays), serology of Immunoglobulin (IgM, IgG, IgG avidity), antigen detection (pp65), viral culture, dried blood spots (DBS), tissue biopsy, and functional CMV assays such as immunoassays (Flow cytometry, QuantiFERON-CMV and ELISpot Assays) CMV smear are included. Molecular techniques, especially PCR-based methods, provide rapid, sensitive and specific detection of Cytomegalovirus DNA or RNA from various samples and remain the gold standard for congenital, transplant and immunodeficient patients. Serology and antigen-based testing provide complementary information, but have limitations in distinguishing active infection from latent infection. Recommended laboratory algorithms emphasize the integration of timely sampling, quantitative viral load monitoring, and patient immune status to optimize clinical decision making. Despite progress, there are still research gaps in universal newborn screening, prenatal prediction, standardization of viral load thresholds and analysis harmony. Future directions aim to improve molecular and immune-based diagnostics, standardize laboratory workflow, and improve patient care in high-risk populations.

Keywords: CMV, congenital CMV, PCR, viral load , QuantiFERON-CMV, ELISpot, molecular diagnostics.

List Of Abbreviation: CMV (Cytomegalovirus), NAAT (nucleic acid amplification testing), PCR (polymerase chain reaction), TMA (transcription-mediated amplification), HIV (human immunodeficiency virus), DBS (Dried blood spots) , IHC (immunohistochemistry), ELISA (enzyme like immunosorbent assay),NGS (next-generation sequencing).

1-Introduction

Cytomegalovirus (CMV) is part of the Herpesviridae family and is one of the most common viral diseases globally (1,2). Cytomegalovirus (CMV) is a large, enveloped double-stranded DNA virus with an icosahedral capsid, tegument proteins that modulate host responses, and surface glycoproteins that mediate cellular entry and immune evasion (3). It has lifelong latency after the original infection and can be reactivated during immunosuppression (4). The global prevalence of CMV varies from 40% to more than 90%, depending on socioeconomic conditions, population density, and regional healthcare practices (5). Despite its prevalence, CMV infection poses a significant diagnostic and therapeutic challenge, especially in vulnerable groups such as newborns, pregnant women, organ transplant recipients and HIV/AIDS patients (6). Laboratory diagnosis of cytomegalovirus (CMV) infection is based on several universal approaches, with the decision largely influenced by the patient's immunological status, clinical presentation, and the specific type of infection suspected. CMV causes asymptomatic infection in most immunocompetent hosts, but it can cause severe disease in fetuses (congenital CMV), neonates, and infants. Immunocompromised persons (transplantation, oncology) (7). Accurate laboratory diagnosis is essential for early treatment decisions (eg, valganciclovir in symptomatic neonates), infection control and epidemiology (8). Recent worldwide agreements and studies have improved diagnostic criteria for prenatal, neonatal and transplant contexts (9).

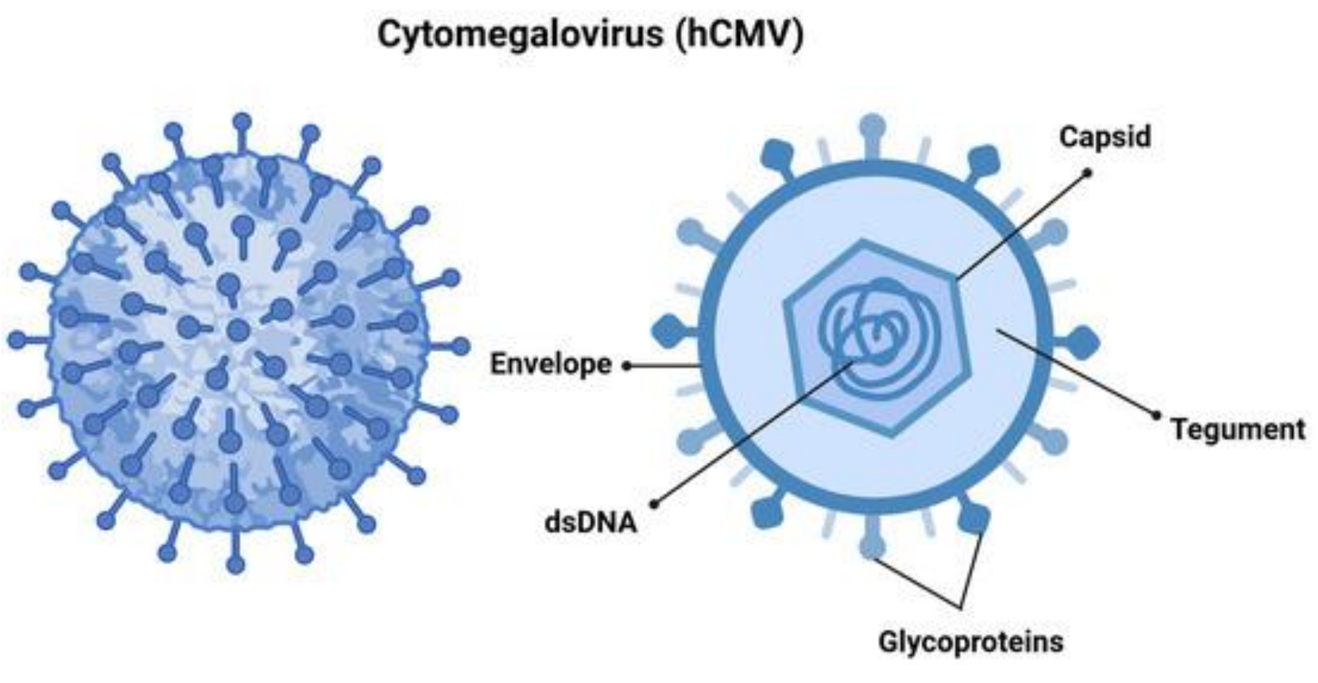

Figure 1: shows the structure of human cytomegalovirus and its components (2).

2. Diagnostic CMV Modalities.

2.1 Nucleic Acid Amplification CMV Tests.

Commonly, cytomegalovirus (CMV) infection is diagnosed by nucleic acid amplification tests (NAAT), such as conventional and real-time by polymerase chain reaction (PCR), which identify CMV DNA directly from patient samples (10). This technique was extremely sensitive and specific, and has largely supplanted classical viral culture due to its faster and more accurate findings. PCR can be used on a variety of clinical specimens depending on the patient's condition, including blood, plasma, urine, saliva, cerebrospinal fluid, amniotic fluid, and tissue biopsies, Although cerebrospinal fluid and bronchoalveolar lavage are sometimes used (11). PCR is usually reserved for severe or unusual cases in immunocompetent individuals, whereas the method of quantitative PCR was routinely used in immunocompromised patients (such as HIV-positive individuals and patients on chemotherapy) to detect CMV viremia and detect viral load, thereby guiding antiviral therapy and assessing treatment efficacy (12). CMV PCR technology in transplant patients allows early detection and follow-up of viral replication, guides preventive antiviral medication to avoid CMV disease, reduce complications and improve transplant outcomes (13). CMV PCR was used to rapidly identify congenital infection in infants, ideally within the first 21 days of life, using saliva or urine samples. A positive PCR result indicates active CMV replication, while a negative result essentially eliminates active infection, provided sample quality and timing are adequate (14). Overall, PCR-based NAAT was the most accurate, rapid, and clinically useful technique for identifying CMV in all patients (14,15).

2.2. TMA chemistry.

These molecular techniques use reverse transcriptase and RNA polymerase enzymes called transcription-mediated amplification (TMA), an isothermal nucleic acid amplification technique that amplifies rapidly from CMV RNA. It allows rapid, highly sensitive and specific detection from blood, urine, saliva, amniotic fluid and tissue. It additionally supports CMV viral load monitoring in transplanted and immunocompromised individuals, allows early diagnosis of congenital or active infection, and overcomes the limitations of culture and serology by detecting low-level CMV viral replication (16,17)

2.3 CRISPR-based CMV.

The clustered regularly interspaced short palindromic repeats (CRISPR-based CMV) detection uses Cas enzymes (such as Cas12 or Cas13) directed by CMV-specific RNA sequences to recognize viral DNA or RNA, and upon recognition, the triggered Cas enzyme cleaves a reporter molecule resulting in a detectable fluorescent or colorimetric signal; The method provides ultra-sensitive, rapid and highly specific detection, and can be used with blood, saliva, urine or dried blood spots, making it suitable for point-of-care testing and potentially universal newborn screening testing (18,19).

2.4 Serology (IgM, IgG, IgG avidity).

One of the most common laboratory techniques for determining the immunological status of certain patient groups and identifying CMV primary infection remains serological testing. Avidity indices for IgM, IgG and IgG are the main methods for estimating and evaluating antibody levels (20).

CMV IgM

These are clearly visible in early infection, usually within 1–2 weeks of viral interaction and may persist for several months. The presence of IgM often indicates recent infection; However, false positive findings may occur due to cross-reactivity with other herpes viruses or non-specific immune activation. Furthermore, CMV reactivation or reinfection can result in a secondary IgM antibody response, complicating interpretation—especially in immunocompromised individuals or transplant recipients (7,21).

CMV IgG

Antibodies appear soon after IgM and persist throughout life, serving as a reliable laboratory marker and indicator of latent CMV virus infection. In clinical practice, IgG serostatus plays an important role in matching transplant donor and recipient, with CMV-seronegative recipients of CMV-positive grafts (D+/R-) having a greater risk of severe infection (22).

CMV IgG avidity

The test measures the functionality and development of IgG antibodies. During initial infection, the antibodies have low avidity (weak adhesion strength), which eventually increases to high CMV avidity IgG within 2–4 months. Therefore, low IgG avidity in the presence of IgM indicates a recent primary infection, while high avidity indicates a previous infection or reactivation rather than primary CMV virus infection (23,24,25).

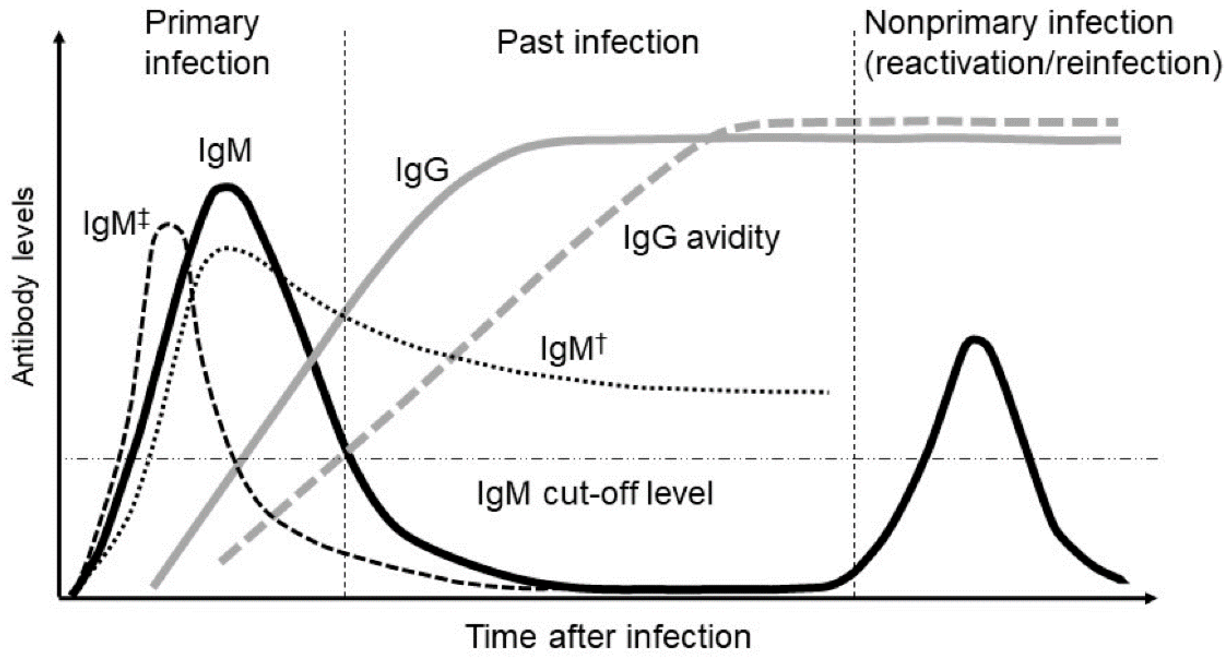

Figure2 : Relative variations in cytomegalovirus (CMV) IgM , IgG and IgG avidity levels over time after initial CMV infection (26).

2.5 Direct antigen detection and culture

- **Antigenemia (pp65) assays:**

CMV antigenemia testing is a semiquantitative technique for detecting current CMV infection, particularly in immunocompromised individuals such as solid organ and hematopoietic stem cell recipients. The test relies on the detection of CMV pp65 antigen, an essential lower matrix phosphoprotein (PP65) expressed in peripheral blood leukocytes during replication of CMV virus infection (27,28).

- **Viral culture (shell vial):**

A rapid and efficient viral culture technique involving clinical samples (eg urine, saliva, blood, tissue) centrifuged onto fibroblast monolayers to enhance viral penetration. After a short incubation period (1–2 days), cytopathic effects begin to appear after one to three weeks (29). Viral immediate-early antigens are detected using CMV-specific immunofluorescent antibodies, enabling early detection of active CMV infection, especially in immunocompromised individuals, newborns and transplant recipients, with faster results and greater specificity than the classical method (conventional culture). But this method is less sensitive than PCR viral load levels (30).

2.6 Dried blood spots (DBS) and newborn screening technologies.

Specimens of dried blood spots may be a useful approach for newborn screening for congenital cytomegalovirus (CCMV) infection. During a routine newborn check-up, a small blood sample is taken (often by heel prick) on filter paper (31). CMV DNA is then identified using real-time PCR performed immediately on DBS cards. This technique allows for retrospective testing, is less invasive, and can be easily integrated into current newborn screening programs. DBS-based CMV detection is useful for detecting neonates with asymptomatic or subclinical CMV infection who may be at risk for sensorineural hearing loss or neurodevelopmental delay, allowing immediate antiviral treatment and continuous monitoring. Nevertheless, it is a useful, scalable and cost-effective technique for population surveillance in medical virology laboratories (32,33,34).

2.7 Newer molecular / genomic methods.

New molecular and genomic methods for CMV detection, including digital droplet PCR (ddPCR) and next-generation sequencing (NGS), which provide rapid, highly sensitive and specific detection methods for viral genomic CMV from blood, urine, saliva, CSF or tissue; enables accurate measurement of viral load to monitor therapy and predict disease progression; recognize mixed infections and viral genotypes; detection of antiviral resistance mutations (eg, UL97, UL54) in immunocompromised or transplant patients; and are invaluable for screening newborns for congenital CMV, directing antiviral therapy, and tracking epidemiology or outbreak strains. These methods outperform traditional culture and serology methods in terms of speed, accuracy and clinical utility (35).

2.8 Biopsy-based CMV.

When a patient is suspected of having tissue invasive CMV disease, a tissue sample is taken and tested by immunohistochemistry (IHC) for CMV antigen and histopathology to look for the classic "owl's eye" inclusion bodies. PCR on biopsy increases sensitivity by detecting even small amounts of viral DNA. Particularly in immunocompromised individuals where CMV establishes a strong hold, this triad helps distinguish between true CMV organ involvement and benign viral cameos (36).

2.9 QuantiFERON-CMV.

Without direct detection of viral DNA or active infection, QuantiFERON-CMV is a functional whole blood assay that uses CMV-specific peptides to induce CD8+ T cells to release interferon-gamma, which is measured by ELISA and reflects the patient's CMV-specific cellular immunity. It is used to evaluate the risk of CMV reactivation, to guide prophylaxis in transplanted or immunocompromised patients, and may be affected by immunosuppression, lymphopenia and peptide coverage (37,38).

2.10 ELISpot Assays.

Although they do not directly detect active viral replication, ELISpot tests for CMV are extremely sensitive functional tests that stimulate peripheral blood mononuclear cells with CMV-specific peptides, causing individual CMV-specific T cells to secrete interferon-gamma that is captured on a membrane and visible as spots (39). This enables accurate quantification of cellular immunity, assessment of the risk of CMV reactivation in transplanted or immunocompromised patients, monitoring of immune recovery and evaluation of vaccine responses (40).

2.11 Flow cytometry.

Flow cytometry to detect CMV is similar to staining white blood cells to highlight any latent CMV agents. Technically, peripheral blood leukocytes are permeabilized and stained with fluorescent monoclonal antibodies that target CMV antigens such as pp65 or immediate-early antigens. Then the flow cytometer laser scans each cell like a nightclub bouncer, looking for viral proteins (41). This method provides rapid turnaround time (same-day results), can determine the percentage of infected cells and viral antigen load, and is particularly useful in transplant or immunocompromised patients to guide antiviral therapy. But it requires fresh samples, trained technicians and can miss lower-level infections than PCR, so it is used as an early warning system rather than the ultimate detective (42).

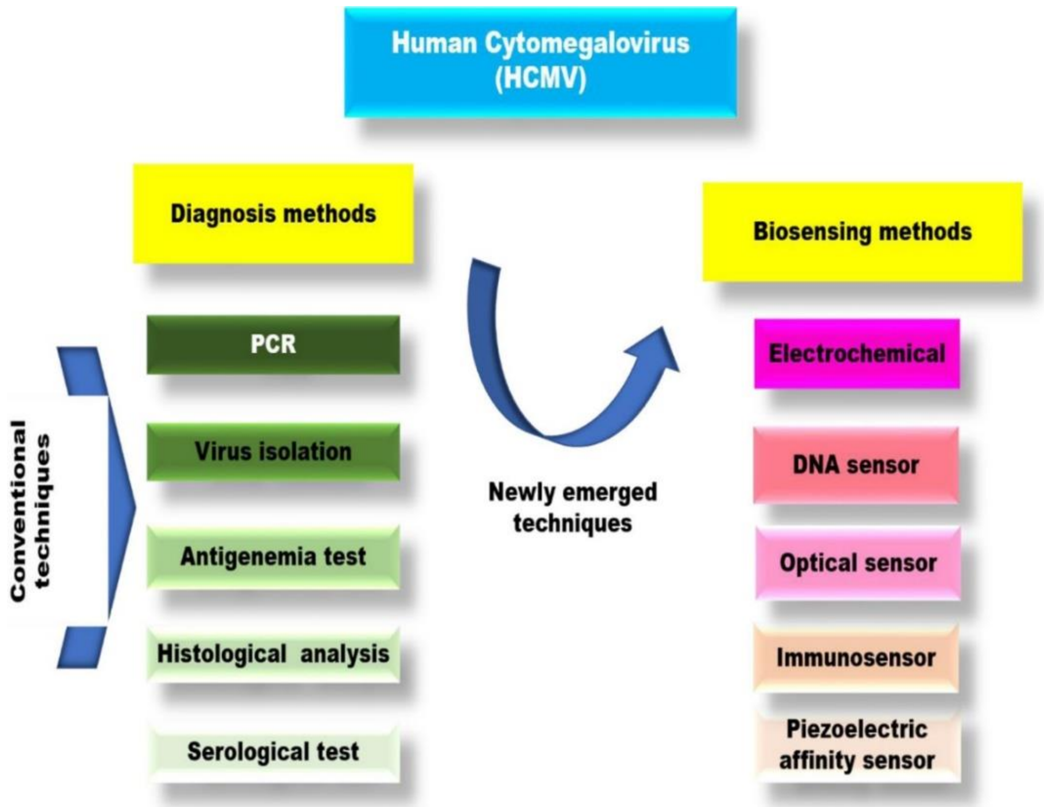

Figure 3: Various techniques of diagnosis of (HCMV) **(43).**

3. Timing and specimen recommendations.

3.1 Congenital CMV (neonates)

Blood samples or dried blood samples may be used for supplemental evaluation or population-based programs, but screening after three weeks should be carefully considered, as it may indicate postnatal transmission, especially in infants who were breastfed. For congenital CMV, testing should be done within the first two to three weeks of life to differentiate between congenital and postnatal infection (44). Saliva is used for initial screening (high sensitivity, easy to obtain), and urine is analyzed for confirmation as gold standard methods. PCR is the most common laboratory diagnostic technique (14).

3.2 Prenatal diagnosis

More serology (IgM, IgG and IgG avidity) performed early in pregnancy, together with PCR analysis of amniotic fluid collected via amniocentesis after 21 weeks of gestation and at least 6–8 weeks after maternal infection is the preferred method and the gold standard for the identification of fetal CMV infection. Ultrasound can provide supportive findings of congenital disease. These tests are recommended for prenatal CMV confirmation in cases of possible primary infection in the mother (45).

3.3 Immunocompromised / transplant patients

Early surveillance for CMV should be performed within the first three months after transplantation or during immunosuppressive therapy to aid in antiviral treatment and prevent progression of the condition. CMV testing should be performed continuously for preventive detection, especially in immunocompromised or transplanted individuals. Commonly, whole blood or plasma is used for quantitative PCR to measure CMV viral load, antigenemia testing (PP65) in leukocytes for early detection of existing infection, and tissue biopsy when organ involvement is possible (15,46).

4. Test performance and limitations

1. **PCR sensitivity and specificity:**

   The most accurate and gold standard molecular method for early diagnosis and monitoring of viral load in congenital, transplanted and immunodeficient patients was PCR for the detection of CMV due to its exceptionally high sensitivity (≈95-100%) and specificity (≈90-100%), which allows accurate detection of CMV virus in blood counts in urine DNA despite too low number of viruses in the blood of the virus. make capable. Saliva, CSF, amniotic fluid and tissue (47).

2. **Serology limitations:**

   IgM can remain elevated for months and maternal IgG crosses the placenta, complicating neonatal diagnosis, and serologic testing for CMV (IgM, IgG, and IgG avidity) is simple and useful in determining prior exposure or CMV infection. However, it cannot distinguish between active and latent disease, can generate cross-reactivity or false positives from false negatives in immunocompromised patients, and provides little information about viral load or current replication, limiting its use in congenital and transplant settings (23,48).

3. **Viral load variability:**

   Variation in CMV viral load, test efficiency and limitations: PCR-based assays (qPCR, ddPCR) are highly sensitive and specific, but they may show interassay variability, require standardization of viral load thresholds, and may not be able to differentiate between latent and active CMV infection. In addition, serology and antigenemia tests are limited by delayed antibody response, transient positivity, and low sensitivity in newborn or immunocompromised patients (49). CMV viral load can vary depending on sample type, time of collection, immune status of the patient and antiviral therapy, all of which can affect interpretation (50).

4. **Dried Blood Spot (DBS)**

   Performance, limitations, and sensitivity of CMV viral detection by DBS testing: The sensitivity of CMV detection by these methods in newborn blood was lower than urine or saliva PCR, especially in individuals with low viral load, and may lead to false negative results (51). The effectiveness of the test depends on the type of analysis, sample quality and DNA extraction efficiency. In addition, DBS is unable to accurately measure viral load or differentiate between congenital and postnatal CMV viral infections (52).

5. Recommended laboratory algorithm (practical workflow)

A. Suspected congenital CMV (newborn with symptoms or positive screening)

The recommended laboratory algorithm for suspected congenital CMV is outlined as follows: When a newborn shows symptoms or if the screening result was positive, collect urine or saliva during the first two to three weeks after birth for PCR-based CMV DNA detection (also known as the gold standard); If the drug is started, monitor viral load measurements and monitor hearing/neurological evaluation (53). Remember that samples taken after three weeks may reflect postnatal infection rather than congenital CMV (54).

B. Maternal suspected recent infection during pregnancy

For mothers who have recently had CMV infection during pregnancy, the following laboratory algorithm is recommended: IgG avidity testing together with maternal serology for CMV IgM and IgG should be performed if previous infection is suspected. Low IgG avidity with positive IgM indicates primary CMV infection, which requires ultrasound observation for fetal abnormalities and amniocentesis PCR (via amniocentesis) after 20–21 weeks' gestation and approx. 6–7 weeks after maternal infection to confirm fetal CMV (55). Follow-up serology helps determine the time of infection, and PCR quantification can inform clinical decisions (56).

C. Immunocompromised patient (transplant)

Recommended laboratory strategy for CMV in the immunocompromised or during transplantation: Evaluate donor/recipient status by performing baseline CMV serology (IgG) before transplantation; For early detection and viral load-directed preventive treatment, routine screening for CMV DNAemia after transplantation using quantitative PCR (whole blood or plasma); If PCR is not available, use the antigenemia test (pp65) (57). Evaluate genotypic resistance testing in cases of refractory or recurrent infection, and modify monitoring frequency according to immunosuppressive level, graft type, and prior CMV exposure (58).

6. Quality assurance and lab operational considerations

Operational Variables and Quality Assurance Controls for CMV Virus Detection: Standardized sampling, proper storage and transport (such as freezing or refrigeration for DNA stability), and rapid processing are essential for accurate CMV testing (59). Laboratories should participate in proficiency testing and external quality assessment programs, use validated molecular assays (qPCR, ddPCR) with internal controls, ensure inter-assay calibration to consistently report viral load, meet biosafety procedures due to infectious samples, and maintain documentation and traceability for clinical decision-making of efficacy variability and type class variability, analysis type. Limitations should be kept in mind. Negative/positive (60).

7. Conclusion

Cytomegalovirus (CMV) remains an important pathogen in newborns, pregnant women and immunocompromised patients, whose diagnosis requires a multidisciplinary laboratory approach tailored to the patient population and the clinical context. Molecular methods, especially PCR and new technologies such as TMA, ddPCR, NGS and CRISPR-based assays, facilitate rapid, sensitive and specific detection of CMV DNA or RNA in various sample types and are the gold standard for congenital and transplant-associated CMV surveillance. Serology (IgM, IgG and IgG avidity) remains useful in assessing the timing of previous exposure and maternal infection, but cannot reliably predict active infection or severity. Antigen detection (pp65) and viral culture provide additional tools, especially in immunocompromised patients, while functional immunoassays such as flow cytometry, QuantiFERON-CMV and ELISpot allow assessment of CMV-specific cellular immunity and risk of reactivation. Early, properly timed sampling—especially in neonates and transplant recipients—is essential for accurate diagnosis and treatment guidance. Despite progress, significant gaps remain in assay standardization, viral load thresholds, prenatal prediction, and universal newborn screening, highlighting the need for continued research, harmonization of laboratory algorithms, and integration of new molecular and immune-based diagnostics to optimize CMV management in the population.

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