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SVEUČILIŠTE U RIJECI
ODJEL ZA BIOTEHNOLOGIJU

Diplomski sveučilišni studij
Biotehnologija u medicini

Ema Belužić

Uvođenje kvantitativnog PCRa za određivanja broja novosintetiziranih MCMV

Diplomski rad

Rijeka, 2023

Mentor: doc. dr. sc. Ljerka Karleuša, dipl. ing. bioteh.

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In front of the Committee:

1. Assoc. prof. dr. sc. Igor Jurak

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3. Doc. dr. sc. Ljerka Karleuša

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Abstract

In the research on virology, accurate and sensitive methods for detecting viral pathogens are essential. This study presents comparative analysis of two commonly used techniques, quantitative PCR (qPCR) and plaque assays, for the detection of murine cytomegalovirus (MCMV). Cytomegalovirus (CMV), a member of the Herpesviridae family, causes medical complications in immunocompromised individuals including congenital defects, post-transplant graft rejections and cardiovascular diseases. CMV infection is complex due to its ability to establish latency in its host and capacity to establish lifelong infections. Understanding its dynamics, especially in immunocompromised individuals, is important. The paper presented shows the efficacy of qPCR as a tool for MCMV quantification, presenting its capability to detect viral DNA even in latent or non-active states. Our findings show a considerable growth in sensitivity when using qPCR compared to the conventional plaque assay. qPCR yielded higher viral particles concentrations and demonstrated advantages in terms of precision and quantification accuracy. qPCR sensitivity allows the detection of minimal quantities of MCMV genome, giving us a better understanding of the virus's latent phase and improving our ability to measure viral dynamics. Furthermore, qPCR's ability to provide results with a faster processing time and reduced subjectivity compared to plaque assays presents it as a useful tool for research and clinical applications. The study underlines the potential of qPCR to improve MCMV detection and quantification, showing a better approach in studying the virus, evaluating antiviral treatments, and eventually improving our ability to treat CMV-related diseases.

Keywords: cytomegalovirus, murine cytomegalovirus, plaque assay, quantitative PCR

Sažetak

U istraživanjima u području virologije, precizne i osjetljive metode za otkrivanje virusnih patogena su esencijalne. Ova studija predstavlja komparativnu analizu dviju često korištenih tehnika, kvantitativnog PCR (qPCR) i testova plakova, za detekciju mišjeg citomegalovirusa (MCMV). Citomegalovirus (CMV), član obitelji Herpesviridae, uzrokuje medicinske komplikacije kod imunokompromitiranih pojedinaca uključujući urođene defekte, odbacivanje presatka nakon transplantacije i kardiovaskularne bolesti. CMV infekcija složena je zbog svoje sposobnosti uspostavljanja latencije u svom domaćinu i sposobnosti uspostavljanja cjeloživotnih infekcija. Razumijevanje njegove dinamike, posebno u imunokompromitiranih osoba, iznimno je važno. Predstavljeni rad pokazuje učinkovitost qPCR-a kao metode za kvantifikaciju MCMV-a, prikazujući njegovu sposobnost detekcije virusne DNA čak i u latentnom ili neaktivnom stanju. Naši rezultati pokazuju značajan porast osjetljivosti pri korištenju qPCR-a u usporedbi s konvencionalnim testovima plakova. qPCR detektirao je veće koncentracije virusnih čestica i pokazao prednosti u pogledu preciznosti i točnosti kvantifikacije. Osjetljivost qPCR-a omogućuje otkrivanje minimalnih količina MCMV genoma, što nam daje bolje razumijevanje latentne faze virusa i poboljšava našu sposobnost mjerenja dinamike virusne infekcije. Nadalje, sposobnost qPCR-a da pruži rezultate s bržim vremenom obrade i smanjenom subjektivnošću u usporedbi s analizama plakova predstavlja ga kao koristan alat za istraživanja i kliničke primjene. Studija naglašava potencijal qPCR-a za poboljšanje detekcije i kvantifikacije MCMV-a, predstavljajući bolji pristup u proučavanju virusa, procjeni antivirusnih tretmana i na kraju poboljšanju naše sposobnosti liječenja bolesti povezanih s CMV-om.

Ključne riječi: citomegalovirus, mišji citomegalovirus, test plakova, kvantitativni PCR

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1. Introduction

1.1. Herpesviruses

Herpesviruses are a large family of DNA viruses that infect a wide variety of animals, including humans. There are over 200 herpesvirus species that have been identified, and are divided into three subfamilies, *Alpha, Beta* and *Gammaherpesvirinae* (1). Human herpesviruses cause medical complications in immunocompromised individuals, including cold sores, oral and genital herpes, cytomegalic inclusion disease, and are associated with tumors (2). Main characteristic of herpesvirus biology is asymptomatic infection with ability to establish latency in its host, causing a disease during primary infection, potentially followed by reactivation (3). There are members of human herpesviruses in all three herpesvirus subfamilies, including herpes simplex virus type 1, type 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV) of alphaherpesviruses, cytomegalovirus (CMV) and human herpesviruses 6 and 7 of betaherpesviruses; and Epstein-Barr virus (EBV) and human herpesvirus 8 of gammaherpesviruses (2).

Herpesviruses share common specific virion morphology. The average herpesvirus' virion measures between 150 and 200 nm in diameter. They are double-stranded DNA (dsDNA) viruses. Linear dsDNA of herpesviruses is tightly packed into an icosahedral capsid coated with an amorphous layer, called tegument, a layer composed of different viral proteins. The outermost layer, following the tegument, is a viral envelope; layer comprised of both cellular proteins and virus-encoded glycoproteins, considered to be essential for viral entry in the cell. Glycoproteins gB, gH, gL, gM, gN, gO are generally considered to be conserved within the herpesviruses. gB, in its homotrimeric form, as well as heterodimer of gH and gL play an important role in the primary

mechanism of fusion between the virion envelope with the host cell membrane during the infection. On the other hand, heterodimer comprised of gM and gN is involved in virion assembly (4).

1.2. Cytomegalovirus

Human cytomegalovirus (HCMV, also known as human herpesvirus 5) is a member of the betaherpesvirus subfamily and causes asymptomatic infection in about 70% of the world population. HCMV is thought to be frequent opportunistic infection in fetuses causing congenital defects; in patients with HIV infection and transplant recipients, HCMV is linked to post-transplant graft rejection and cardiovascular diseases (5). HCMV shows broad cell tropism; with ability to infect high range of cell types including smooth muscle cells, epithelial cells of gland and mucosal tissue, vascular endothelial cells, fibroblasts, macrophages, dendritic cells and hepatocytes (6). A characteristic feature of HCMV infection is the ability to evade host immune system and avoid elimination. HCMV has developed complex methods to evade hosts immune system by disrupting antigen presentation to CD4 and CD8 T lymphocytes, as well as downregulating NK cell-activating ligands. It achieves this by using certain genes that inhibit activation of NK cells, and reducing the presentation of viral antigens by downregulation of MHC-I. Another way of avoiding immune response is induction of latent state of infection (7). HCMV infection is complex, and depending on the infected individual, can be presented in several different ways. In a person with no immunity (or heavily immunocompromised), this virus develops primary infection within the first contraction. The second type of infection comes when the virus develops latency, from which it may reactivate. The third type of infection is reinfection, which occurs when a person who has previously been infected becomes

infected again, despite having natural immunity, due to being in contact with an infectious individual.

The most commonly used model for CMV studies is murine cytomegalovirus (MCMV), due to difficulties associated with studying HCMV pathogenesis in humans, using CMVs trait of highly restricted host range. The biological characteristics of MCMV infections in mice correspond to the characteristic of HCMV infection in humans. Similar to HCMV, MCMV induces severe infection in immunocompromised hosts, and it is used for studying viral pathogenesis, latency, immune evasion, and disease mechanism (8). One of advantages in using MCMV as a model in research is the duration of its replication cycle. While HCMV full replication cycle lasts up to 96 hours in total, MCMV replication cycle is much shorter and lasts from 24 to 48 hours with the same spatio-temporal classes of genes/proteins; only within different time segments (9). Shorter replication cycle is beneficial in studying CMV life cycle and virion maturation as well as other cellular mechanisms affected by the infection itself.

1.2.1 CMV virion structure

Being a member of herpesvirus family, cytomegalovirus virion consists of genome, capsid, tegument and envelope (Figure 1). The cytomegalovirus virion measures from 120 to 200 nm in diameter. Its DNA is linear double-stranded molecule measuring from 220 (HCMV) to 235 (MCMV) kbp and has about 230 genes encoding 54 membrane proteins and around 25 membrane glycoproteins present in the virion envelope. Host proteins have also been identified as components of virions. Some of the host proteins include intercellular transport factors suggesting their role in vesicle trafficking in viral egress (10).

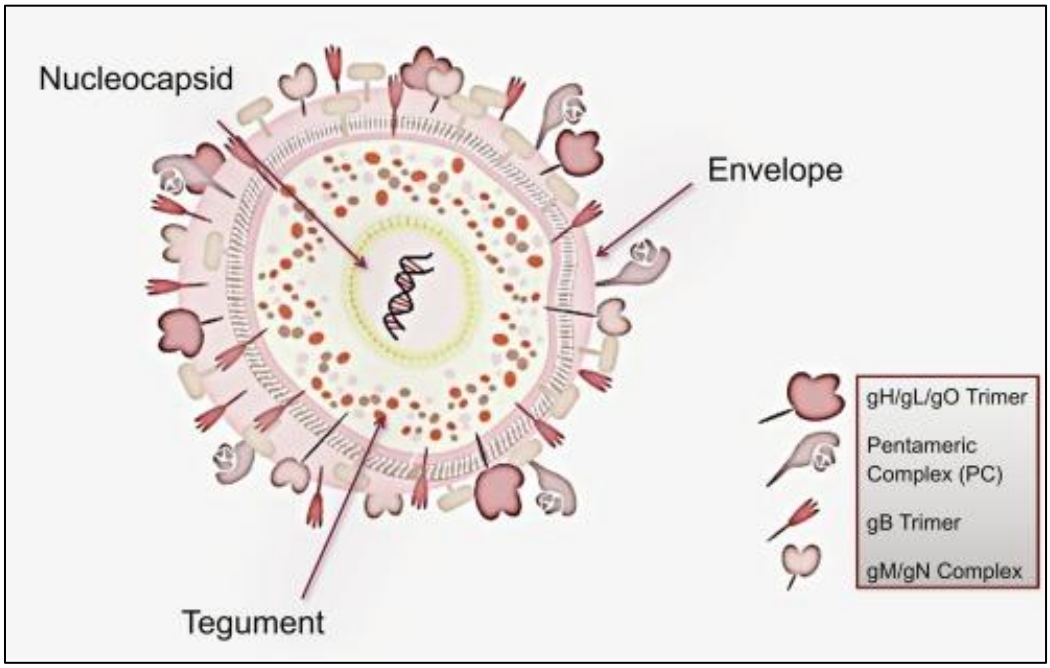

Figure 1. Cytomegalovirus virion. CMV virion has linear double stranded DNA (dsDNA) packed into capsid surrounded by protein-rich layer, the tegument. Surrounding the tegument is an envelope, phospholipid bilayer containing virus-encoded glycoproteins including, among other proteins, gB trimer, gH/gL/gO trimer, gM/gN dimer and gH/gL/UL128/UL130/UL131 pentamer (11).

1.2.2. CMV cell entry

Cytomegalovirus can infect a wide variety of cells within its host. The main targets for viral replication of CMV are epithelial cells, endothelial cells, smooth muscle cells and fibroblasts. Those cells that support the replication of viruses are called permissive cells. On the other hand, cells in which replication of virus is restricted are called nonpermissive cells, and infection of nonpermissive cells will not result in synthesis of new virions. In both permissive and nonpermissive cell types, viral binding and cell entry are quick and efficient (7). During the process of entering a cell, the outer surface of infectious viral particles, containing glycoproteins that interact with receptors

on the host cell, can trigger either fusion of virus with the cell, or endocytosis of the viral particle into the cell (Figure 2). CMV entry into fibroblasts involves fusion at the plasma membrane, while entry into epithelial and endothelial cells involves endocytosis and endosomal system, requiring endosomal pH for fusion (12). CMV uses various glycoprotein complexes to initiate its entry into host cells. Three major glycoprotein complexes, namely gB homotrimer, as well as gM/gN and gH/gL heterodimers, play key roles in this process. These complexes act in consecutive manner; with the gM/gN complex being responsible for initial binding to the host cells, the gH/gL heterodimer promotes binding to the receptors on the cell surface, and the gB homotrimer mediates the final fusion between cell membrane and infective viral particle. The binding of gH/gL complexes to receptors causes conformational changes that activate gB complex, enabling it to carry out the fusion of the viral and host cell membranes (13). CMV encodes two distinct gH/gL complexes, each with different functions. The trimeric complex, composed of gH/gL and gO (gH/gL/gO), initiates viral entry into all cell types, particularly fibroblasts. In addition, CMV also uses a pentameric complex formed by gH/gL and small glycoproteins UL128, UL130, and UL131. This pentameric complex expands the cell tropism of CMV, allowing infection of epithelial cells, endothelial cells, leukocytes, and dendritic cells, while not affecting fibroblasts. CMV's capacity to infect different cell types can be connected to its usage of several host surface receptors and co-receptors that facilitate viral entry. The gH/gL/gO complex specifically infects fibroblasts binding to the platelet-derived growth factor receptor (PDGFR), which is absent on epithelial cells. The pentameric complex, on the other hand, targets neuropilin-2 (Nrp2), allowing for effective infection of epithelial and endothelial cells (13). Following virus-cell membrane fusion, the tegument proteins enter the host cytoplasm and interact with the hosts' microtubule machinery for rapid translocation of viral nucleocapsid into the nucleus where the transcription of viral genome and its replication begins.

At the same time, proteins from the viral tegument are released into the infected cell, or more precise, into its cytoplasm, and are translocated to different parts of the cell to block the initial immune response and control the spatio-temporal expression of viral genes.

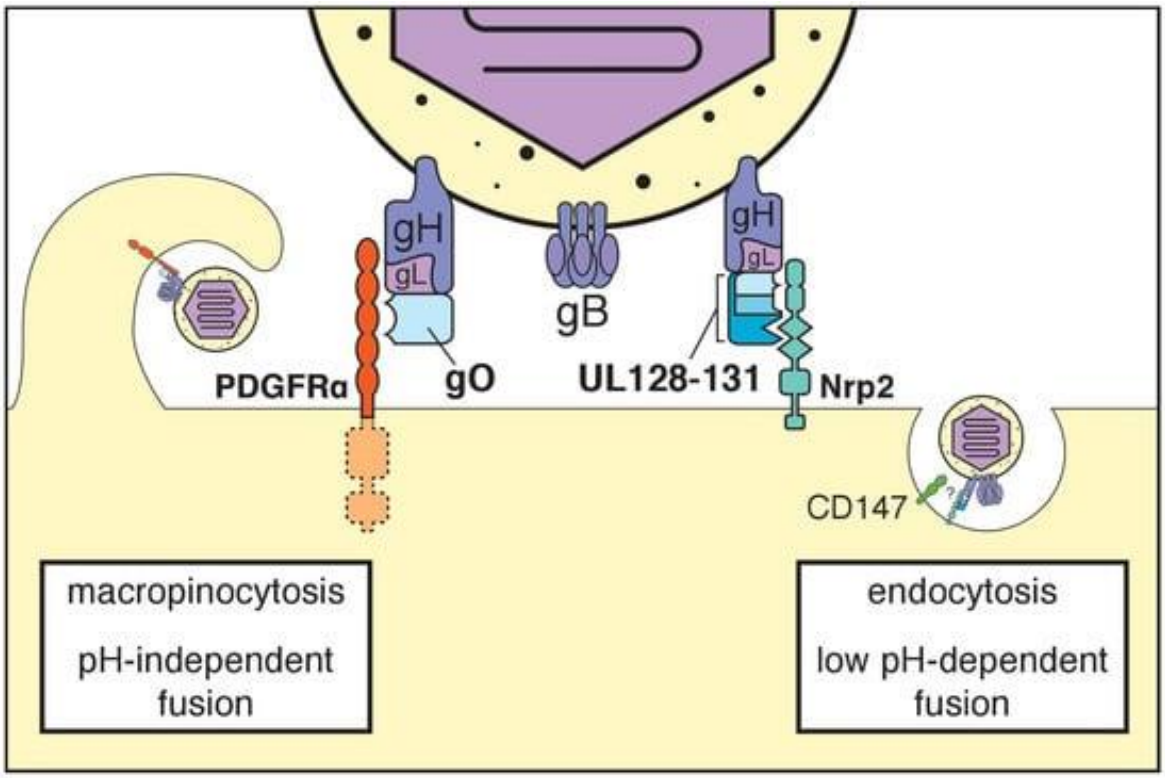

Figure 2. Cytomegalovirus cell entry. The gH/gL/gO complex causes infection of fibroblasts by binding to the platelet-derived growth factor receptor-α (PDGFRα) it enters the cells through micropinocytosis in pH-independent manner. The pentameric complex formed by gH/gL and small glycoproteins UL128, UL130, and UL131 targets neuropilin-2 (Nrp2), facilitating infection of endothelial and epithelial and cells using endocytosis in a low pH-dependent manner. On the other hand, it has been proposed that gB acts as viral fusogene and does not bind to membrane receptors (4).

1.2.3. CMV infection

When CMV enters permissive cells, it causes a reaction of temporally regulated gene expression, characteristic for herpesvirus lytic infection(14). The binding and interaction of CMV glycoproteins with their receptors is key for initiation of intracellular signal transduction cascade, resulting in changes in both cellular gene expression and expression of viral genes and proteins that in return regulate cellular metabolism and signaling. There are three main classes of the viral proteins that each regulate different feature of CMV infectious cycle (15). There are immediate early (IE), early (E), and late (L) viral proteins, their names pinpointing the time period in the viral infection when they are predominately expressed. IE gene expression starts within 1 hour post infection (hpi) and initiates (H)CMV gene expression. Some of the IE genes are the major IE UL122/123 genes (IE1 and IE2) and auxiliary genes. There are two subclasses of E genes including E genes and E-L genes. the expression of E genes depends on the presence of IE proteins. Expression of E genes occurs from 4 to 8 hpi and expression of E-L genes from 8 to 24 hpi (7). The replication, inversion, and packaging of the (H)CMV genome occur in the nucleus of infected cells and viral DNA synthesis begins 16 hours after infection. The transcription of late proteins starts 24 hours after infection and is strictly dependent on DNA replication. In some cells, including nonpermissive cells, arrest in viral gene expression can occur and therefore restrict viral replication. In MCMV infection replication cycle is significantly shorter. IE phase is limited to 1-2 hpi and E phase is limited to 2-16 hpi. The DNA synthesis, L phase, begins around 16 hpi corresponding with the initiation of late genes' transcription. The general temporal profile of viral protein expression as well as simplified changes in the cellular endosomal system during infection with MCMV are shown in Figure 3.

IE proteins are essential in initiating CMV gene expression, by acting as transactivators and stimulators of the overall viral gene expression (7). Also, they have an impact on host cell physiology, regulating expression of many host cell genes. IE protein(s) will regulate the expression of both early and late genes, later during infection progress. Early genes encode mostly non-structural proteins, including those involved in immune evasion, repair enzymes and viral DNA replication factors. They are involved in the development of molecular systems that remodel host cell immune response and reorganize host cell organelles and endosomal system, as well as cytoskeleton. Early proteins are also involved in the initiation of new virion formation, firstly in nucleus and then in cytoplasm.

The late proteins are the final stage of gene products produced during CMV replication. Functions of the late proteins are predominantly structural regarding virion assembly and morphogenesis (7).

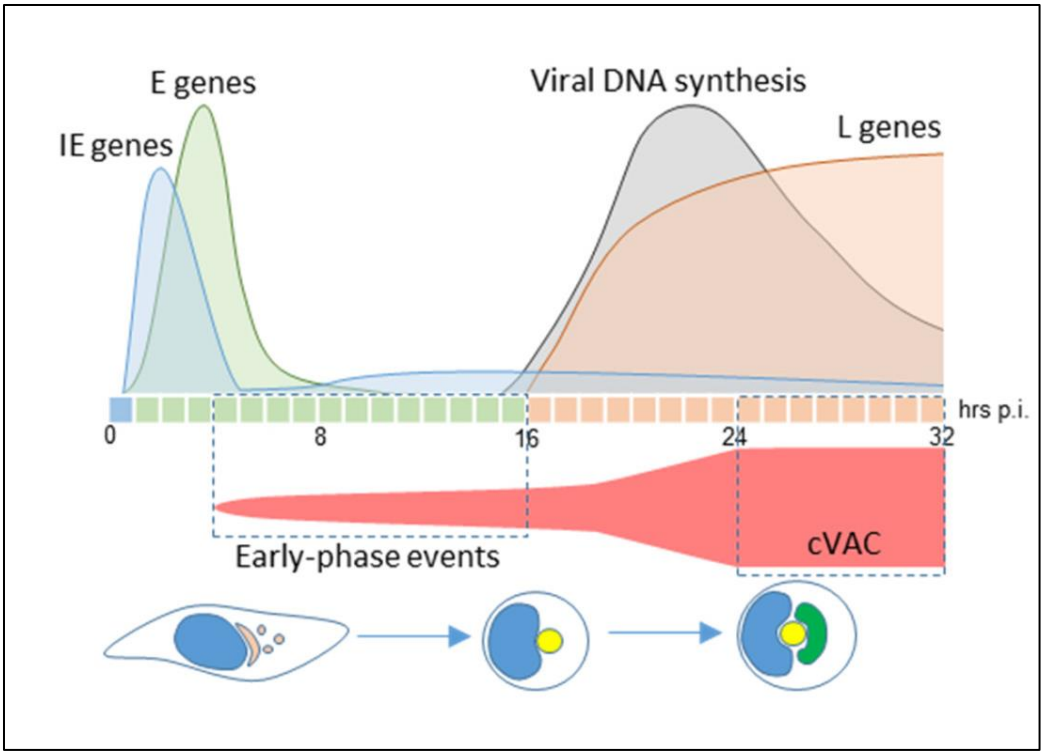

Figure 3. Timeline of MCMV protein expression and important cellular changes. MCMV genes are expressed in different time phases: immediate early (IE), early (E), and late (L). The gene expressions affect cellular functions during the viral replication cycle. The reorganization of membrane organelles that form the cytoplasmatic virion assembly compartment (cVAC) is shown in red. During the early phase of the infection, the endosomal recycling compartment and Golgi reorganize, forming a juxtanuclear structure that becomes the center of the cVAC. When viral DNA synthesis and expression of late genes is happening, viral tegument proteins accumulate in the cytoplasm, and viral glycoproteins form a cap around the core (16).

1.2.4. CMV virion formation and cellular egress

CMV capsid formation and viral DNA packaging begin in the cell nucleus, where capsids are formed and packed with viral genetic material. They obtain primary envelope at the inner nuclear membrane, while budding into nuclear membrane's lumen. This process is called primary envelopment (17). Upon entering the cytoplasm, through the outer nuclear membrane, newly formed capsids lose their primary envelope and are transferred to the cytoplasmatic viral assembly compartment (cVAC) where the final virion assembly takes

place. The cVAC is a newly formed structure in cytoplasm derived from several, already existing, cellular compartments (18). During the first 24 hours following infection of MCMV, the host cell endosomal system and key cytoplasmic components are remodeled to become the cVAC. The cVAC formation depends on virus DNA replication and on expression of several viral late genes. The first stage of cVAC formation in MCMV infection starts during the E phase of infection from 3 to 16 hpi and the structure further expands during L phase of infection from 16 to 24 hpi. In the cVAC, newly synthesized virions obtain most of their tegument, they become enveloped, and are then transported to the cell membrane for release from the cell.

The cVAC is arranged as cylindric structure, with more or less concentric structures surrounding the microtubule organizing center situated in the middle. Golgi apparatus, ER-to-Golgi intermediate compartment, and trans-Golgi network (TGN) form the outer cylinder (19). Markers for early endosomes (early endosomal antigen 1, EEA1) and markers for endosomal recycling compartments (i.e., Rab11) are situated more closely to the structural center. These markers indicate viral reorganization of early endosomal and endosomal recycling system in biogenesis of the cVAC (Lučin et al., 2020).

The nucleus is bent around one side of cVAC, creating kidney-like nucleus typical for CMV infections. Nuclear membrane becomes porous with increased distance between its inner and outer membrane.

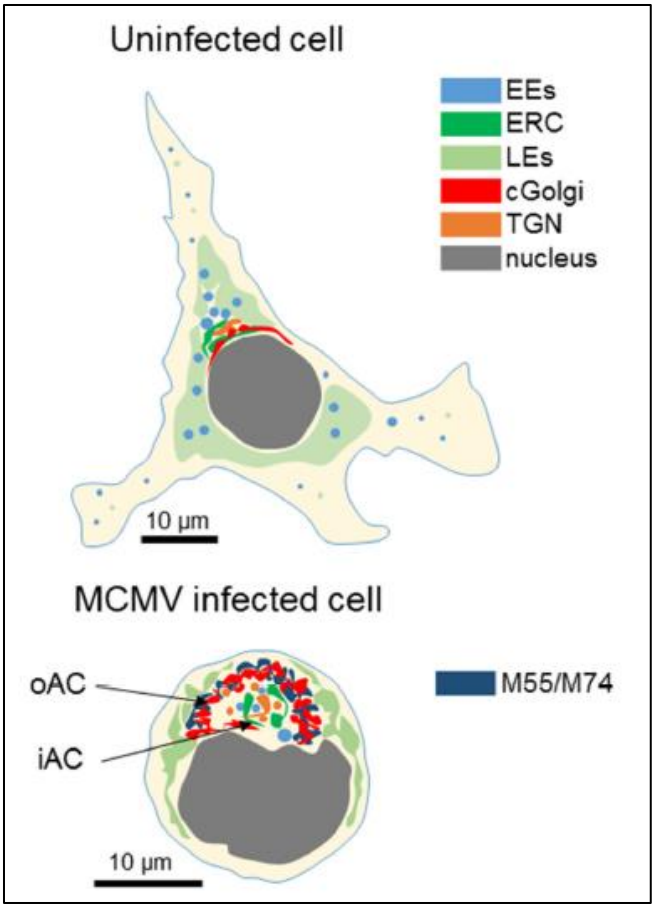

Figure 4. Organelle reorganization during MCMV infection in Balb-3T3 cells. Schematic representation shows formation of cVAC in MCMV infected cells. It is assembled by reorganizing cell endosomal system and different cytoplasmic compartments. After remodeling, markers of early endosomes and endosomal recycling compartments are found in the center of cVAC, and Golgi apparatus is found in the outer ring of cVAC (20).

Following successful envelopment, new mature virions are transported to the cell membrane for viral egress which is still poorly understood. There are several factors that are thought to be involved, mainly connected with the regulation of secretory vesicle transport (exocytosis) of CMV. Rab GTPases (i.e., Rab3 and Rab27) that control intracellular transport pathways, regulate transport and docking of secretory vesicles on the plasma membrane (21). Following vesicle docking to the plasma membrane, SNAP/SNARE complexes are involved in membrane fusion, shown by upregulated expression of

Syntaxin 3 (STX3), SNARE protein that can initiate vesicle plasma membrane fusion, in the CMV infection. Syntaxin3 can also be found in cVAC. Knockdown of STX3 reduces the production of infectious virions (22). However, complete inhibition of those pathway regulators does not eliminate CMV egress completely, suggesting that there are alternative pathways of viral egress. Because of the complexity of endosomal system there are many alternative pathways that virus can exploit for virion egress when primary pathways are inhibited. More research is needed for understanding cellular trafficking events in both cytomegalovirus and general herpesvirus egress.

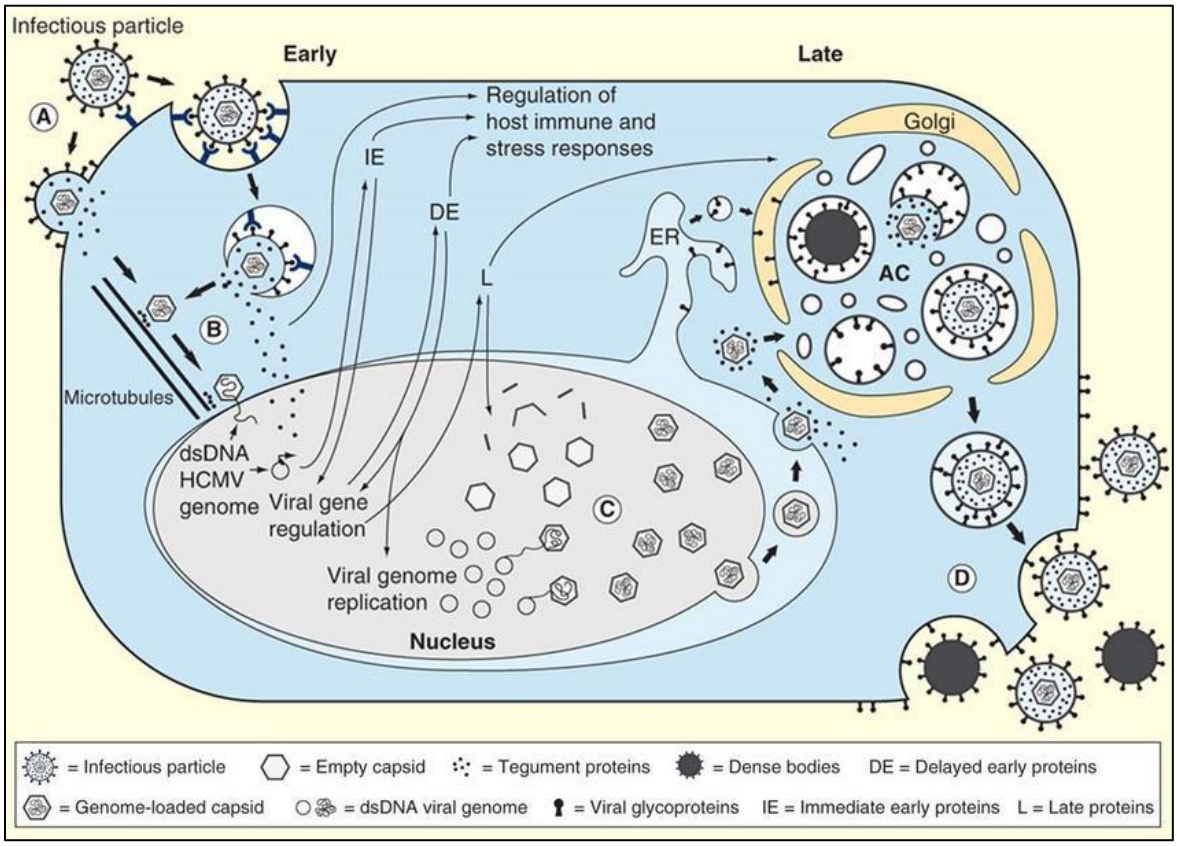

Figure 5. Human cytomegalovirus life cycle. (A) Infectious particle enters the host cell by binding to different membrane receptors and capsid and tegument proteins enter the host cytoplasm, while the envelope is left behind. (B) Capsid is translocated to the nucleus where the genome is transferred. Tegument proteins are released in the cytoplasm where they block the initial immune and stress responses, as well as control viral gene expression initiating expression of IE genes and subsequently E

genes. Early genes are responsible for initiation of viral genome replication and late genes expression. (C) Capsid formation and DNA packaging starts in nucleus after which capsids are trafficked to the cVAC where the final assembly and envelopment takes place. (D) Mature virions are then transported toward the cell membrane for egress (15)

1.3. Detection of MCMV

Detection and quantification of CMV, or more precise infective CMV units, in laboratories is done by standard plaque assay, still considered to be "gold standard" in determining viral concentrations of infectious lytic viruses, including herpesviruses. In plaque assay, murine embryonic fibroblasts (MEF) are used as a vessel for determination of the number of infectious MCMV units. Plaque assay is used for determination of amount of the plaque forming units (PFU) – infectious MCMV units in a sample. In the completely confluent cell culture, MCMV-infected cell will lyse and spread infection to adjacent cells, that will also undergo lytic infection. The plaque, or "clear patches" where there are no cells, will form in the area where infected cells previously existed. Plaques are counted visually, each one corresponding to one infectious particle (23). However, calculated PFU does not represent with certainty that each plaque is formed by initial infection of a cell by a single viral particle, because of CMV's unique feature to form multicapsid virions. Multicapsid virion is infectious product comprised of several complete and mature viral capsids (with genetic material) that are enveloped by single membrane, which would then be perceived as single plaque in a plaque assay (24).

Some other limitations to plaque assay are that analysis and counting of plaques mostly depends on the experience of individual performing the assay and their, non-voluntary, potential, subjectivity. In addition, this assay is incredibly time-consuming, starting with MEF culture preparation to MCMV infection time and it usually takes two whole weeks from culturing MEFs until counting of the plaques. Additionally, plaque assay is not the most fitting method for fast screening of large panels of samples, while researching influence of different cellular of chemical parameters to virus production and/or synthesis.

Quantitative Polymerase chain reaction (qPCR) is an alternative method that can be used in detection and quantification of MCMV and is generally more

sensitive and precise than plaque assay. Also, this is a completely objective method, given that it is performed correctly. qPCR is a method predominantly used in evaluating gene expression, in MCMV context, to determine starting viral load, as well as detection of the chosen viral protein acting as a functional infection marker (in this case we will use gene coding for m86 protein).

Quantitative PCR amplifies and quantifies specific regions of the DNA template, from the viral DNA using PCR technology. The amount of detected amplified DNA is proportional to the initial amount of viral DNA in the sample (25). Therefore, the qPCR can be used as more specific and sensitive method to determine number of MCMV DNA copies in a sample (26). In this work we will be determining the number of copies of MCMV M86 gene. The M86 gene encodes for the large capsid protein of MCMV particle. Given that there is only one copy of this gene in one viral genome, one detected gene copy of M86 will correspond to one viral DNA in processed sample.

2. Aim of the study

Accurate quantification of MCMV is important for future research. It helps researchers to precisely monitor viral loads and measure the efficacy of antiviral treatments or vaccines. Better results in virus quantification help in understanding the virus's dynamics and pathogenesis contributing to the development of more targeted therapies. The aim of this study is to test the protocol for quantification of MCMV viral particles in infected primary mouse embryonal fibroblasts (MEF) using quantitative PCR and compare its accuracy and sensitivity over standardly used plaque assay method. Additionally, we will detect and examine MCMV infection during different time points by detecting IE protein (IE1) and L protein (m55) localization in infected mouse fibroblasts.

Our hypothesis is that using qPCR for detection and quantification of MCMV in infected cells is both a more precise and more accurate method than plaque assay.

3. Materials and methods

2.1. Materials

2.1.1. Media and buffers

- DMEM medium for Balb3T3 cells and MEF cells: DMEM medium, 2mM L-glutamine, 1x105 U/L penicillin, 0.1 g/L streptomycin sulfate, and 5% (MEF) or 10% (Balb 3T3) (v/v) fetal calf serum (FCS).

- Methylcellulose medium: 0.022 g/ml methylcellulose, 26 mM sodium bicarbonate (NaHCO3), 10x concentrated MEM, 3% FBS, $1x10^5$ U/L penicillin, 0.1 g/L streptomycin sulfate.

- Paraformaldehyde: 40 g/L paraformaldehyde; 0.01 M sodium hydroxide (NaOH).

- Permeabilization buffer – Tween 20 (1%): 1% Tween-20 in PBS.

- Mounting medium- Mowiol: 2.4 g Mowiol; 6 mL glycerol; 0.2 M Tris; 2.5% DABCO.

2.1.2. Primary and secondary antibodies

- anti-IE1- clone CROMA 101, mouse IgG_1 antibody, produced at the Department of Physiology, Immunology and Pathophysiology
- anti-m55- clone m55.02, mouse IgG_{2b} antibody, Center for Proteomics
- anti-mouse IgG_1 goat antibody conjugated with Alexa Fluor 594, Molecular Probes, Invitrogen

- anti-mouse IgG_{2b} goat antibody conjugated with Alexa Fluor 594, Molecular Probes, Invitrogen

2.1.3. Cell lines

Immortalized adherent Balb 3T3 mouse fibroblast cells as well as primary mouse embryonal fibroblast (MEF) cells were infected.

2.1.4. Murine cytomegalovirus

In this study we used recombinant murine cytomegalovirus (MCMV) Δm138-MCMV (ΔMC95.15) with deletion of fcr1 (m138) gene encoding for Fc receptor like viral protein.

2.2. Methods

2.2.1. Balb 3T3 cell culture

Adherent Balb 3T3 cells were grown in plastic petri dishes in DMEM medium, supplemented with 2 mM L-glutamine, 1x 10^5 U/L penicillin, 0,1 g/L streptomycin sulfate and 10% FCS. Cells were grown at the temperature of 37°C and 5% CO_2.

2.2.2. Primary mouse embryonal fibroblasts (MEF) cell culture

MEF were grown in plastic petri dishes in DMEM medium, supplemented with 2 mM L-glutamine, 1x 10^5 U/L penicillin, 0,1 g/L streptomycin sulfate and 5% FCS. Cells were grown at the temperature of 37°C and 5% CO_2.

2.2.3. Infection of Balb 3T3 cells with murine cytomegalovirus (MCMV)

Adherent Balb 3T3 grown in 12 well microtiter plates with or without glass slides covering the bottom and infected in cold growth medium with 1 PFU per cell of ΔMC95.15 virus. Cells were centrifuged at 2000 g for 30 minutes (15 minutes on each side) to reach the standard multiplicity of infection (MOI). Cells were then placed in the incubator, and incubated at the standard conditions and analyzed during different time points.

2.2.4. Immunofluorescent microscopy

For immunofluorescent microscopy, adherent Balb 3T3 cells were grown on 12 well plates with sterile glass cover slips added to the bottom of the wells. Cells were washed with PBS and fixated for 20 minutes with 4% paraformaldehyde (PFA) at room temperature. After 20 minutes, cells were washed with PBS three times. For permeabilization we used 1% Tween in PBS for 20 minutes at 37°C. After washing with PBS again, primary antibody in 10% medium was added to cells for 1 hour and 30 minutes at room temperature. Secondary antibody was diluted in 10% medium and then added to the cells for 45 minutes at room temperature in the dark. Cells were washed with PBS between each step. As a final step, we stained the nucleus with DAPI, diluted in PBS for 5 minutes in the dark. Cells were washed again and mounted in Mowiol.

Cells were photographed using epifluorescent Olympus BX51 microscope equipped with DP71CCD camera (Olympus, Tokyo, Japan) with UPlanFL N 40×/0.75 objective with CellX Programe.

2.2.5. Viral sample preparation

Samples were collected at three different time points during the infection: 24-, 48- and 72-hours post-infection. Cell medium was collected in sterile tubes and directly frozen at -80°C. Cells were first removed from the growth surface with 150 µl of trypsin and incubated for 2 minutes on 37°C. After the incubation we neutralized trypsin with medium and collected cells from each sample.

The collected cells were centrifuged at 15000 g for 5 minutes and frozen at -80°C. This was followed by cell lysis protocol in cell samples for DNA extraction using repeated freezing and thawing cycles. We added 1 mL of medium into the collected cell samples and repeated 4 cycles of freezing and thawing. Cells were frozen at -80°C for 20 minutes and then thawed in water bath (37°C) for 5 minutes.

Both the cell supernatant and the cell-derived samples were further used for qPCR and plaque assay.

2.2.5. Plaque assay

Previously prepared MEF cells were grown in 5% growth medium in plastic petri dishes. For plaque assay cells were displaced into 48 well plates and incubated at the temperature of 37°C and 5% CO_2, until full confluency. We prepared dilutions of viral suspension and applied them to the wells in duplicates. Plates were then centrifuged at 2000g for 30 minutes (15 minutes on each side) to increase the efficiency of infection. We returned the plates to the incubator at 37°C for 30 minutes. After 30 minutes, cells were coated with methylcellulose that prevents spreading of the virus through the medium after cell lysis. Prepared plates were then incubated at 37°C and 5% CO_2 for 4 days. For counting the plaques in each well, we used an inverted microscope.

2.2.6. DNA purification

DNA purification was done using NucleoSpin cfDNA XS purification kit (MACHEREY-NAGEL) using high sensitivity protocol for purification of circulating DNA from plasma. We used 220 µl of sample and added 20 µl of Proteinase K. After incubation on 37°C for 10 minutes, binding buffer was added. We vortexed the samples and spined them down. After mixing the samples we loaded lysate and centrifuged samples at 2000g for 1 minute and 11000g for 30 seconds. After centrifuge, membrane was washed 2 times with 500 µl and 250 µl of washing buffer and centrifuged at 11000g for 3 minutes. Lastly, we eluted DNA with 20 µl of Elution buffer, centrifuged samples at 11000g for 1 minute and heated samples at 90°C for 8 minutes to remove residual ethanol.

2.2.7. qPCR

Quantitative PCR was done for detection of MCMV M86 gene with the Applied Biosystems 7300 Real-time PCR system. We used forward primer: GGT CGT GGG CAG CTG (600 nM) and reverse primer: CCT ACA GCA CGG CGG AGA (600 nM). Each 25 µl reaction contained 12,5 µl of SybrGreen PCR Master Mix (2 X), 2,5 µl of each primer, 5 µl of DNAse-free water and 2,5 µl of sample DNA.

2.2.8. Gel electrophoresis

For conformation of standard with known DNA concentration that we used in PCR we added 20 µl of each PCR standard sample to wells in 1,5% agarose gel. For visualization 5 µl of nontoxic Millipore Gel red nucleic acid stain was previously added to samples. For product length conformation 5 µl of DNA

ladder was loaded. The gel underwent electrophoresis for 20 minutes at 100 V. The gel was then examined under UV light to confirm uniform DNA segment size.

4. Results

4.1. Balb 3T3 cells infection and localization of viral markers

The progression of cell infection was monitored across four distinct stages of infection. Samples were collected at various time points, including a control group of non-infected cells, as well as at 24-, 48-, and 72-hours post-infection (Figure 6). During the infection cells were monitored visually using inverted microscope to detect their changes in morphology. Non infected fibroblasts show distinct morphology and are elongated and flat in shape. Starting at 24 hours post infection, cells morphology changes and up to 72 hours post infection all infected cells exhibit round morphology. Rounding of cells is a common change found in virus infected cells (Figure 6).

To determine the infection through immunofluorescent microscopy, specific protein markers with precise transcription timings during the cell infection were labeled. In this study, we used antibodies targeting the IE1 protein, a multifunctional regulatory protein encoded by immediate early genes that initiate transcription in the first hours of infection. The IE1 protein has important roles in viral gene expression, modulation of host cell functions, and evasion of the host immune response. It interacts with transcriptional machinery, promoting the expression of other essential viral genes required for successful infection.

Additionally, we visualized the m55 protein, which represents the glycoprotein B in murine cytomegalovirus (MCMV). The m55 protein shows key functions in viral entry, cell-to-cell transmission, and immune recognition. Its transcription starts during the later stages of infection, specifically during the late phase, and contributes significantly to viral replication, assembly, and pathogenesis. These protein markers were used to confirm and characterize the infection dynamics at different stages of the infection.

In non-infected cells there is no signal for either IE1 or m55 proteins. IE1 protein shows higher levels of expression in the first 24 hours and m55 protein shows highest levels of expression later in infection, during 48 and 72 hours after the initial infection (Figure 6). These results show successful infection of cells with MCMV.

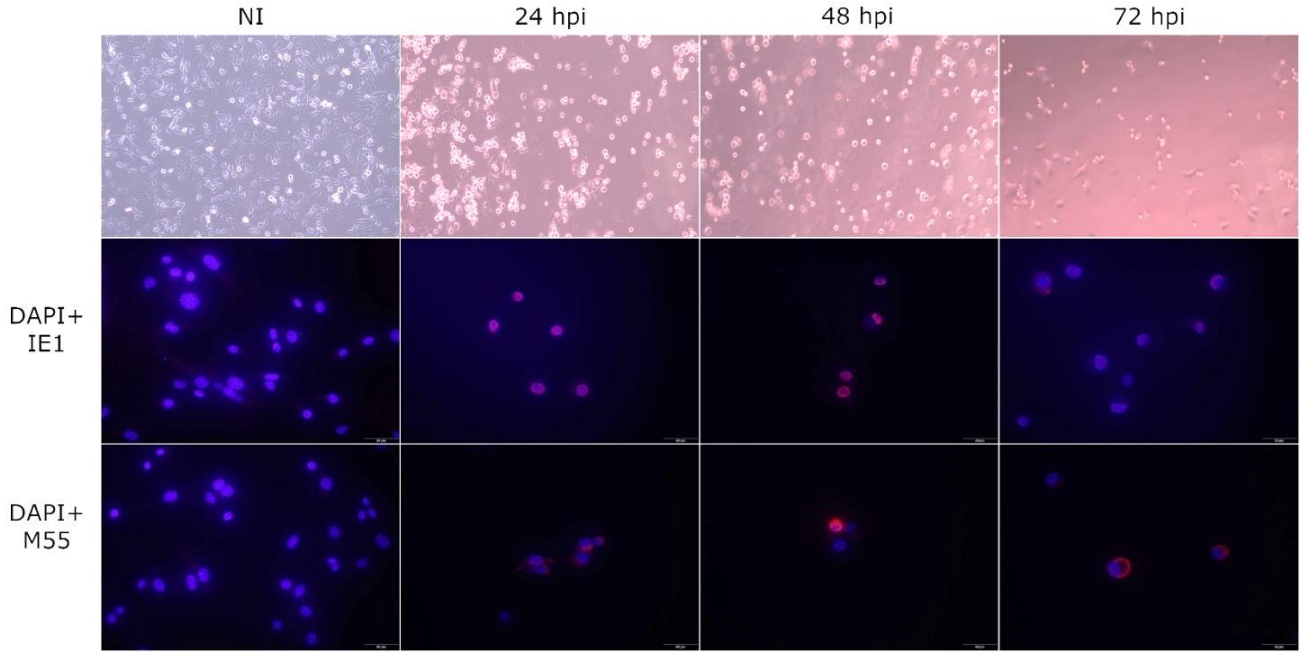

Figure 6. Expression of MCMV infection markers in different time points of infection. The first row represents live cells captured using light microscopy at different stages of infection. Changes in cell morphology are visible starting at 24 hours post infection. Non infected fibroblasts have an elongated shape while infected fibroblast exhibit more rounded morphology. For immunofluorescent microscopy, IE1 protein is visualized using anti-IE1 antibody (CROMA) and anti-mouse IgG_1 antibody conjugated with Alexa Fluor 594 (red). M55 protein is visualized using anti-m55 antibody (m55.02 clone) and anti-mouse IgG_{2b} antibody conjugated with Alexa Fluor 594 (red). All samples were treated with DAPI for better nucleus visualization (blue). In the non-infected cells, there is no expression of these markers. At the 24 hpi we can see expression of IE1 protein and minimal expression of M55 protein. In the later stages of infection expression of IE1 protein is lower and of m55 protein is higher.

During CMV infection, the IE1 protein is primarily found in the nucleus. It is known to accumulate within the nucleus of infected cells, where it serves in several regulatory roles. This nuclear location allows the IE1 protein to interact with the host cell's transcriptional machinery and viral gene promoters, promoting viral gene expression activation. The nuclear localization of IE1 is required for effective viral replication. Also, as it can be seen in Figure 7, IE1 expression is reduced as the infection cycle progresses later than 24 hpi.

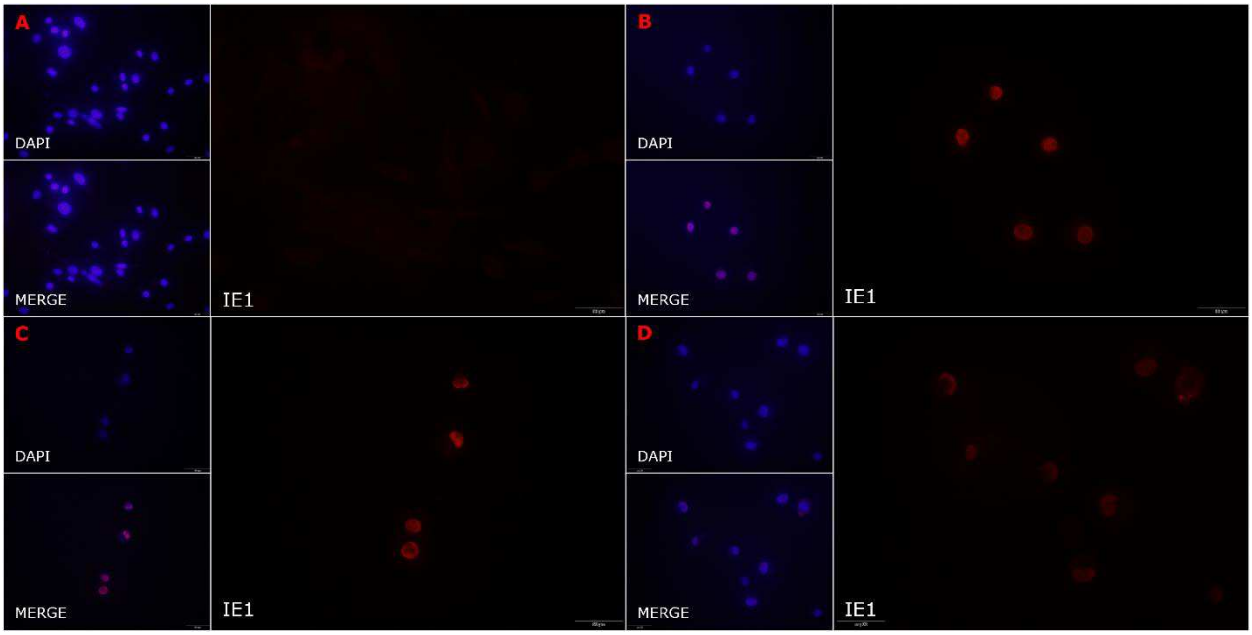

Figure 7. Expression of IE1 marker on Balb 3T3 cells infected with MCMV. (A) In the non-infected cells there is no expression of IE1 protein. (B,C) At the 24- and 48-hour post infection IE1 protein is highly expressed and is mostly localized in host cell's nucleus. (D) At the 72 hours post infection expression of IE1 protein is low. Images were acquired using immunofluorescent microcopy, IE1 marker is labeled with Alexa Fluor 594 (red) and nucleus is labeled with DAPI (blue).

On the other hand, the m55 protein, also known as glycoprotein B (gB), has a different localization pattern. During infection it accumulates in the cell cytoplasm, on the outer part of cVAC. We detected a high expression of m55 after 24 hours post infection localized at perinuclear area where cVAC is

located (Figure 8). As a structural protein, the m55 protein is primarily localized in the viral envelope and is associated with the virion surface. This protein is integrated into the viral envelope during CMV infection, contributing to the structure and function of the viral particle. The m55 protein's location in the viral envelope allows it to play an important role in viral entry, fusion with host cell membranes, and cell-to-cell transmission.

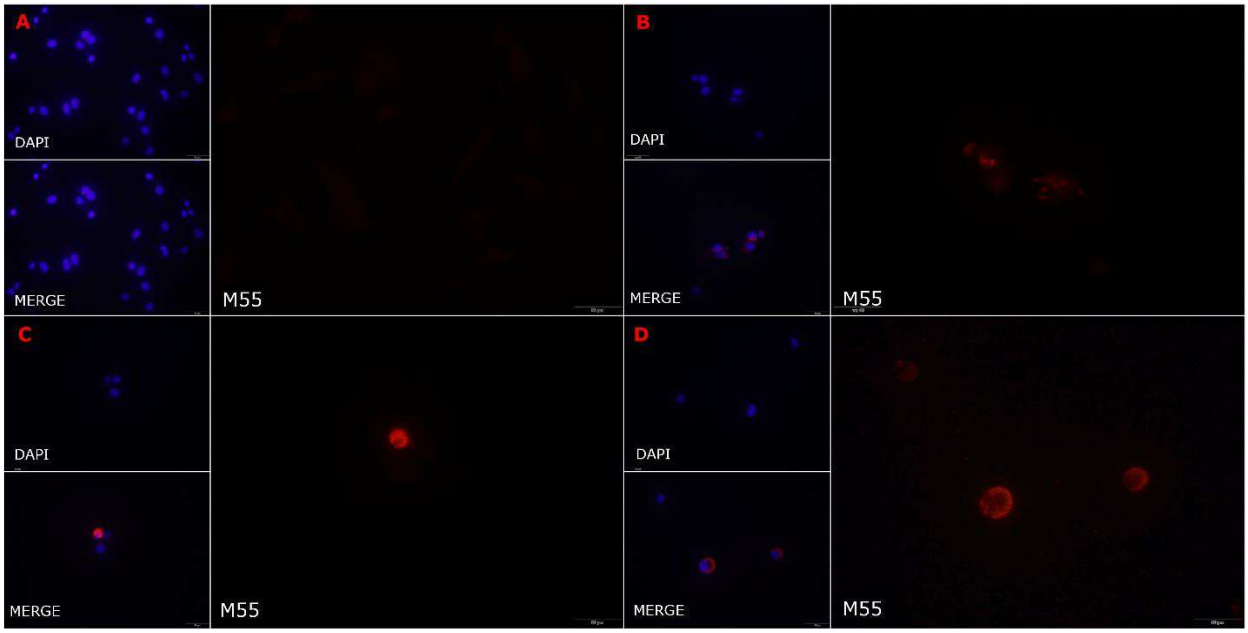

Figure 8. Expression of M55 marker on Balb 3T3 cells infected with MCMV. (A) In the non-infected cells there is no expression of M55 protein. (B) At the 24 hours post infection m55 protein is still not highly expressed, we only detected vague signal. (C, D) At the 48- and 72-hours post infection expression of M55 protein is high. Photographs were captured using immunofluorescent microcopy, m55 marker is labeled with Alexa Fluor 594 (red) and nucleus is labeled with DAPI (blue).

4.2. Plaque assay

We used traditional plaque assay for viral quantification and determining viral titer. Plaque assay is the general golden standard for determining viral titer in lytic viruses. Host cells (in our case MEFs) are grown until full confluency in wells and dilution of viral suspension are added to cells. Virus samples are diluted, and aliquot is added to wells. Cells are then incubated what allows the virus to infect target cells. After incubation, cells are covered with methylcellulose thus forming a gel overlay. The methylcellulose restricts the spread of the new viruses only to the cells adjacent to the originally infected one, resulting in the formation of a circular zone of infected (lysed) cells known as plaque. After a few days, the plaque grows in size, until it becomes visible, and therefore it can be counted. Samples of viral suspension were collected at the 24-, 48- and 72-hours post infection. Samples were prepared in decadal dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} times). Dilutions were prepared in duplicates for higher accuracy. As a negative control we used samples derived from non-infected cells. Cells were incubated for at least 4 days, or until recognizable plaques started forming. Plaques were manually counted using an inverted microscope.

The highest number of plaques counted was, as expected, at 72 hpi and lowest at 24 hpi. In the non-infected samples, there were no visible plaques. In the cell-free samples (cell culture supernatant) the number of formed plaques was significantly higher than in samples of cell suspension. Average PFU/mL calculated in cell-free samples at 24 hpi is $3.6x10^4$, at 48 hpi is $1.5x10^5$ and at 72 hpi is $4.5x10^5$. In contrast, in cell suspension samples average PFU/mL calculates at 24 hpi is $9.5x10^2$, at 48 hpi is $2.3x10^3$ and at 72 hpi is $3.5x10^3$ (Figure 9).In both sample groups the tendency in new virions production over time is shown to be same with higher numbers of PFU at 72- hours post infection.

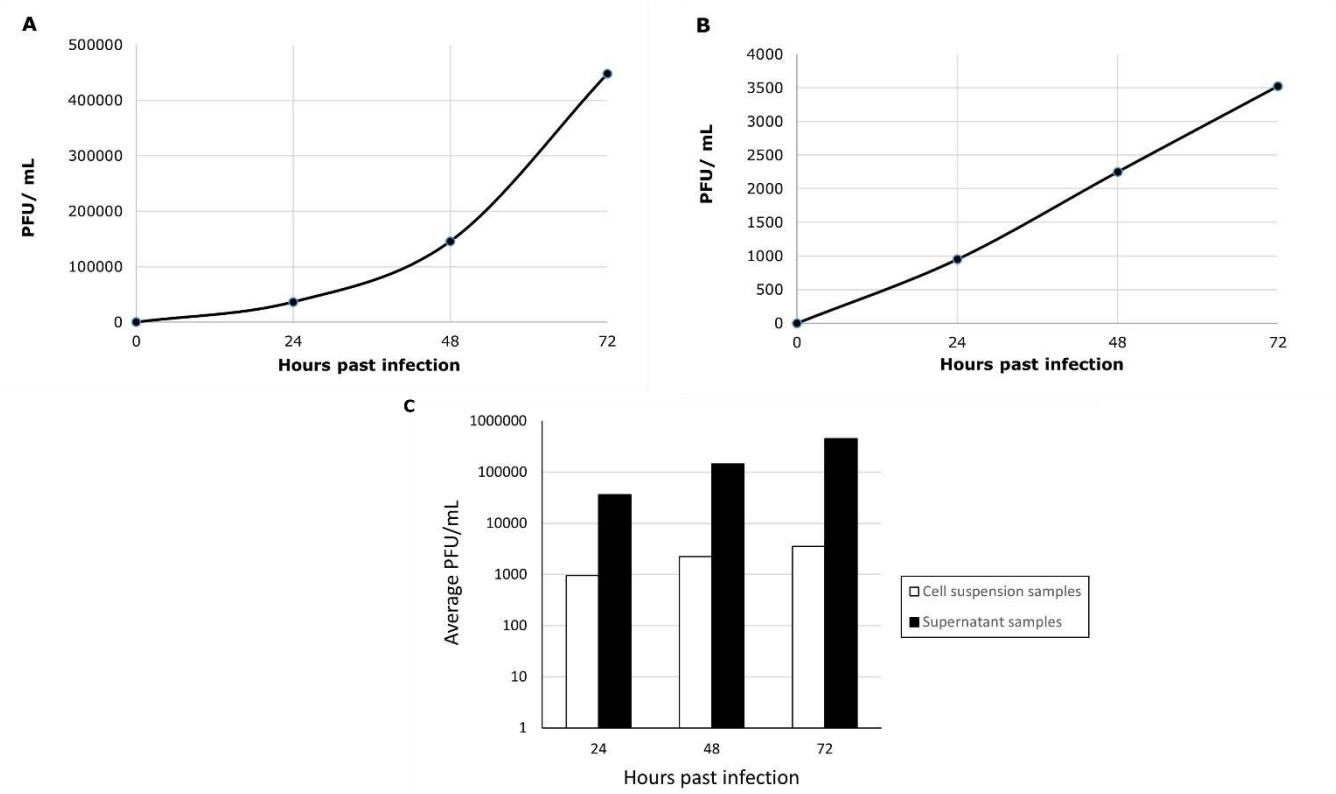

Figure 9. Average PFU/mL detected using plaque assay on supernatant and cell suspension samples. (A) Calculated average PFU/mL of MCMV in samples from supernatant taken at different times after the initial infection using standard plaque assay. (B) Calculated average PFU/mL of MCMV in samples of cell suspension taken at different times after the initial infection using standard plaque assay. (C) Comparison between average PFU/mL from supernatant samples and cell suspension samples showing significant differenece in MCMV levels.

4.3. Quantification of MCMV DNA using qPCR

To quantify the number of MCMV DNA copies present in cell-free supernatant samples and cell suspension samples we used quantitative PCR. As a target gene we used M86 which is known to encode for major capsid protein m86. The m86 protein is the most abundant protein in MCMV capsid, but there is only one copy of it in the entire MCMV genome. During the capsid assembly in nucleus, m86 forms pentameric and hexametric capsomeres, main subunits of capsids. Since there is one copy of M86 gene in the genome of single MCMV

virion when reading the PCR results, one copy of M86 gene will correlate to one MCMV capsid.

4.3.1. Standard curve

In order to be possible for us to determine DNA concentrations in prepared samples, a standard curve was performed (Figure 10). As standard we used DNA sample with known concentration and made 10-fold dilutions of it. Dilutions that we prepared were: 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 10^0, 10^{-1}, 10^{-2}, 10^{-3}. We used the collected data as template to create trendline and determined its equation (Figure 9). Using the standard equation and resulting Ct values of each unknown sample we can calculate concentrations of DNA template in desired samples.

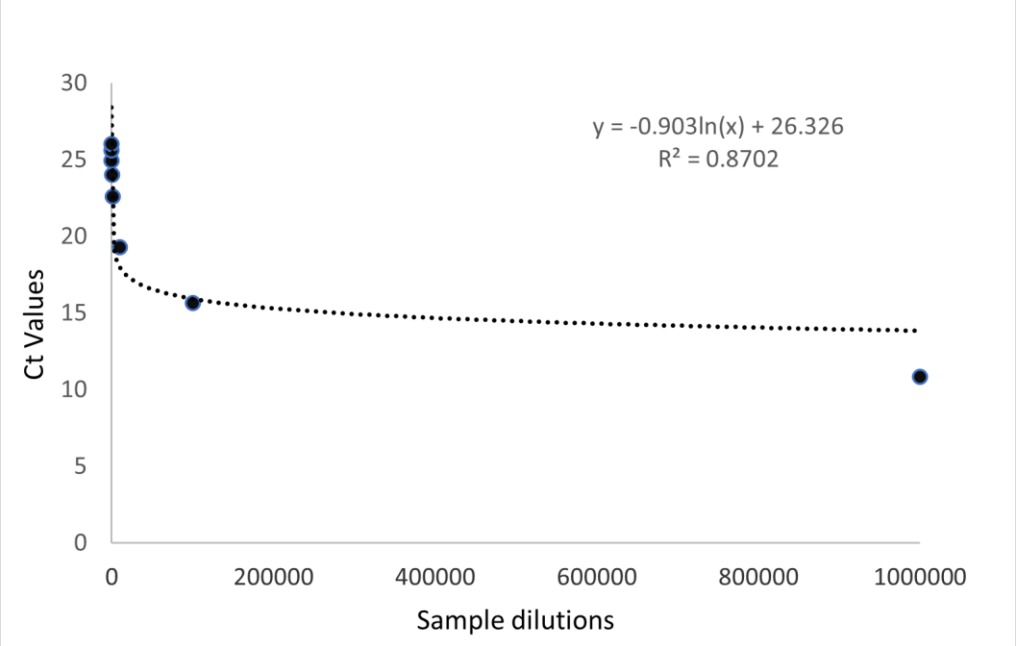

Figure 10. Standard curve of MCMV m86 amplification. Amplification of MCMV gene m86 was measured using qPCR on dilutions of standard samples of known concentrations. On x axis are presented sample dilutions and on y axis are Ct values from qPCR. With higher concentrations of virus Ct values are lower. The equation of the curve shows us the relationship of the values on the x and y axes, with which we will be able to quantify MCMV in the tested samples of unknown DNA concentration. R^2 value

represents correlation coefficient showing what level of confidence we have in predicting the x value based on the given y value.

In order to qualitatively confirm PCR results from our standard, the mentioned samples were loaded to 1,5% agarose gel and underwent gel electrophoresis for 20 minutes. Instead of standard ethidium bromide dye, nontoxic Millipore Gel red nucleic acid stain was used for visualization. Gel was examined under UV light for better visualization and conformation of DNA segment size. All bands showing are uniform in size confirming presence of DNA template in standard samples (Figure 11). As expected, brightness of the bands diminishes when moving toward the samples with smaller concentrations due to serial dilutions.

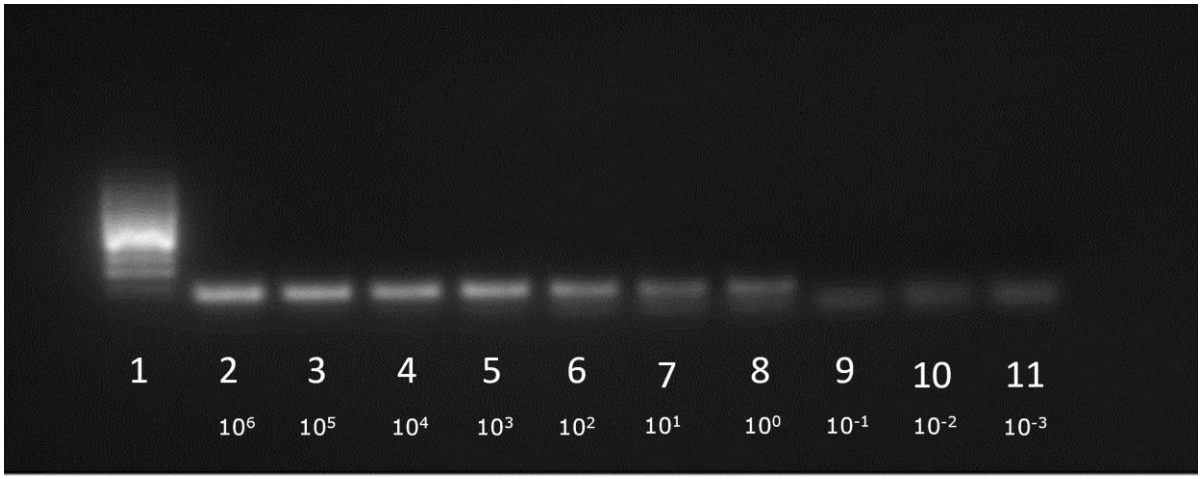

Figure 11. Standard DNA samples on 1,5% agarose gel electrophoresis. Before loading, samples were stained with nontoxic Millipore Gel red nucleic acid stain. The 100 bp – 1500 bp DNA marker was loaded in the first well. Wells number 2 to number 11 contain our standard samples with known concentrations of virus. Samples were prepared as 10-fold dilutions; from 10^6 to 10^{-3}. All bands visible are uniform in size and brightness of each band depends on DNA concentrations in sample which is visible on our gel.

4.3.2. qPCR

Using standard curve equation (Figure 10) and average Ct values for each of the samples, PFU in each sample is determined. The equation for calculation of PFU is derived from standard curve equation where y represents Ct value of sample and x is PFU:

$$y = -0.903 \ln(x) + 26.326$$

When transformed, the resulting equation for PFU calculation is:

$$PFU = e^{\frac{26.326 - Ct}{0.903}}$$

All of the acquired Ct values and calculated average Ct values for each one of the samples are shown in Table 1. Ct values represent value where PCR curve crosses the threshold and detects the fluorescence. The higher the Ct value, the more amplification cycles are required to detect our DNA, implying a lower concentration of template DNA in our sample. We established a consensus, where all Ct values that are higher than 25 are considered to be negative. We analyzed the production of MCMV genomes in two different, independent groups of samples. In each set we measured DNA expression in collected supernatant samples and cell suspension samples, in all the before-mentioned time-points (24, 48 and 72 hpi). Negative controls are considered those derived from non-infected samples. Every sample was analyzed in duplicates for better accuracy.

Table 1. Quantitative PCR results. Samples included in qPCR analysis are divided into two groups corresponding to two independent experiments. In each group, the samples are: supernatant samples (SN) and cell suspension samples (cell). All the samples are processed in duplicates. Analyzed samples were collected at different timepoints after the initial infection including non-infected samples (NI), 24 hours after infection (24hpi), 48 hours after infection (48hpi) and 72 hours after infection (72hpi).

Sample	Ct1	Ct2	Average Ct
NI SN	27.7522	28.3017	28.02695
NI SN	27.906	28.2096	28.0578
24hpi SN	13.7287	14.5248	14.12675
24hpi SN	13.212	13.619	13.4155
48hpi SN	13.076	13.6348	13.3554
48hpi SN	16.465	14.0908	15.2779
72hpi SN	12.8138	13.1388	12.9763
72hpi SN	13.4123	13.61	13.51115
NI cell	36.9364	29.5188	33.2276
NI cell	28.6508	28.0133	28.33205
24hpi cell	17.3826	17.1186	17.2506
24hpi cell	16.7446	17.1665	16.95555
48hpi cell	16	16.5486	16.2743
48hpi cell	15.3252	15.6759	15.50055
72hpi cell	Undetermined	23.3544	23.3544
72hpi cell	16.2257	18.9852	17.60545

PFU values, calculated from obtained standard curve (Figure 10) for all the samples, from independent experiments, both supernatant and cell suspension samples, are presented in Table 2. As expected, the highest PFU measured is supernatant samples as well as in cell suspension samples in 72-hour past infection, and lowest in 24-hour past infection samples. Calculated PFUs for cell suspension samples are clearly lower than in supernatant

samples. The same tendency was also observed in corresponding plaque assay results (Figure 9).

Table 2. Calculated PFU and average PFU samples. The presented PFU/mL of each sample was calculated using the standard curve equation and average Ct value obtained from original qPCR results. Samples are grouped into two separate groups presenting two independent experiments, including samples with supernatant (SN) and samples with cells suspension (cell). Samples taken from different times after initial infection (NI, 24, 48, 72) were tested in duplicates and average values were calculated from values of each duplicate.

Sample	PFU/mL	Average PFU/mL
NI SN	0.152	0.149
NI SN	0.147	
24hpi SN	736518.713	1177777.943
24hpi SN	1619037.173	
48hpi SN	1730460.502	968153.264
48hpi SN	205846.026	
72hpi SN	2633227.743	2044769.672
72hpi SN	1456311.602	

Sample	PFU/mL	Average PFU/mL
NI cell	0.000	0.054
NI cell	0.108	
24hpi cell	23162.199	27637.677
24hpi cell	32113.155	
48hpi cell	68285.363	114574.855
48hpi cell	160864.347	
72hpi cell	26.865	7831.258
72hpi cell	15635.652	

4.4. qPCR vs plaque assay results

For comparison of results of two different methods used for quantification of MCMV, average values for both sets of results are shown in Table 3. Visible differences are observed in sensibility and specific detection of MCMV virions.

Table 3. Average values of PFU/mL detected in plaque assay and qPCR. Average PFU values for all samples were obtained combining sets of results from plaque assay and qPCR. Results present distinctive differences in number of PFU detected.

	NI	24 hpi	48 hpi	72 hpi
Plaque assay	0	18681.25	74050	225762.3
qPCR	0.10197	602707.8	541364.1	1026300

There is a stark difference between average PFU/mL detected in the same samples after using different quantification methods. Calculated PFU/mL using qPCR results are significantly higher than those acquired in plaque assay. A similar trend in virus concentration growth is detected by both methods with the highest concentration being at 72 hours post infection (Figure 12).

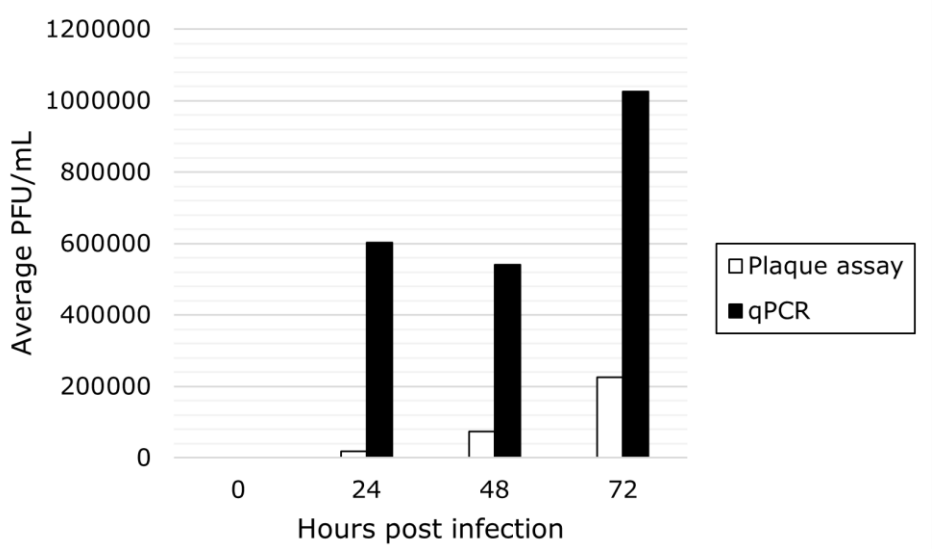

Figure 12. Relation between plaque assay and qPCR results. The difference in detected PFU/ml in same samples using two different quantification methods is shown. Plaque assay and qPCR were performed on samples with unknown viral concentrations taken at different timepoints after infection. Results were presented in two sets from which are average values calculated.

5. Discussion

Cytomegalovirus infection in mice has been recognized as an important study model for researching CMV infection, since it shares the most important infection characteristics with HCMV infection. Although HCMV causes asymptomatic infection in around 70% of general population, HCMV can lead to opportunistic infections in immunocompromised individuals and therefore can result in serious and grave consequences. Using MCMV as a model for research, at the same time lowers the time consumption in studying different aspects of infection as well as reduces possible risks for the researcher as opposed to using HCMV. Due to shorter viral replication cycle it is easier to study CMV specific and crucial events that occur in viral life cycle, that could be connected to its pathogenesis (8).

Accurate and specific methods in detection of newly synthesized MCMV virions in cells and tissues are key in studying potential antiviral mechanisms for infection treatment and drug discovery. In most laboratories, infectious MCMV is usually quantified using plaque assays. They are considered to be "gold standard" in detection and calculations of viral titer. During plaque assay, virus infects MEF cells and through cell lysis it will spread to adjacent cells, which causes formation of visible plaques. Each plaque is then considered as infectious particle and viral titer can be calculated (27). There are several problems when using plaque assay for precise virus detection. Firstly, it is very time consuming considering the time it takes to prepare MEF cell culture, growth of MEF cells, infection with viral suspensions and waiting for the infection to spread and formation of visible plaques. All this together can take over two weeks to complete. Secondly, because of how time consuming, and bulky it is, this method is not ideal when needing to analyze a larger panel of different samples. In addition, results can vary over how much lab experience with plaque assays the person that is counting them has, as well as the quality

of the MEFs (that can change due to their age). Because of that plaque assay can be a very subjective way of detecting viral particles prone to many practical difficulties while performing it. Also, plaque assay detects the ability of MCMV to infect and replicate in cells, which means that it only detects infectious viral particles. Knowing that latency is an important characteristic of CMV infection, plaque assay is not suitable for sensitive quantification of virus because it cannot detect CMV in latent stage (28). Another problem with plaque assay is that calculated PFU doesn't mean that one plaque is formed by infection of singe viral particle (single capsid) because of MCMV specific ability to form multicapsid virions, which differs from other members of herpesvirus family. Multicapsid virions contain several genomes of MCMV, packed in several capsids but enveloped in joint tegument and envelope. Possibility of multicapsid virions can affect the perceived infectivity of the virus (24). Because of that, one plaque does not certainly mean one canonical infectious particle (one capsid with one tegument and one envelope) or one MCMV genome, it only measures that one cell was initially infected with a non-specified infectious particle. Quantitative PCR, on the other hand, will detect every single genome of MCMV present in the processed sample. Using M86 as gene template, which is present in form of only one copy in MCMV genome, we can calculate exactly how many viral genomes are present in our sample of interest. In this experiment, plaque assay and qPCR were used as methods for detecting newly synthesized virions in mouse infected cells to compare overall sensitivity and accuracy of the two methods.

Our data shows that both plaque assay and qPCR successfully detected MCMV particles in samples of interest. In both cases, the highest number of PFU was detected in supernatant samples 72 hours after initial infection (Figure 12). Results also show that there is a clear difference in detected viral concentrations between supernatant and cell suspension samples (Figure 9),(Table 2). After virion maturation and successful envelopment in cVAC in

cell cytoplasm, newly synthesized virions are transported to the cell membrane where most likely, their exocytosis occurs. This is the reason why a higher number of virions is expected outside of a cell (in the supernatant) than in the cell. Viral particles detected in cell suspension represent virions that are still not mature enough for viral egress, or they were on route to release in the moment of sample collection and preparation.

In comparison of the two methods for viral quantification, it was noticed that the average PFU/mL detected were starkly higher using qPCR as quantification method than in plaque assay (Figure 12). The highest average PFU/mL detected by qPCR in 72 hours past infection in supernatant samples is $2.04x10^6$ PFU/mL compared to highest average in plaque assay that is $4.5x10^5$ PFU/mL. These results show that qPCR is more sensitive in detection and quantification of produced MCMV. Our results are in favor with research done by Vliegen et al. which shows that using qPCR for quantification of MCMV particles in different organs is more sensitive than standard plaque assay. The main cause in major difference between qPCR and plaque assay results is that qPCR can detect non-infectious particles as well as infectious viral particles. It detects copies of genome so it can detect viral particles that are not active and are in latent stage of infection, when plaque assay detects only infectious viral particles (28).

Besides the higher sensitivity in detecting HCMV particles, qPCR is a faster method and the whole analysis takes one to two working days, including DNA extraction from samples. An additional advantage of qPCR is that it is easier to analyze larger sets of samples at the same time. Therefore, using qPCR in laboratories would speed up and facilitate research on CMV.

Research could be improved by gaining more experience in executing plaque assay which can be unintentionally subjective. The cause of such high difference in detected PFUs may not only be in the methods alone, but also in conducting researcher’s experience with recognizing and counting plaques.

We also observed some irregularities in 72-hours past infection sample from cell suspension where little to no virus was detected using qPCR (Table 2). One reason for that could be an error in pipetting in preparation of that sample. Regardless of that, significant differences in detection rates of MCMV between plaque assay and qPCR can still be recognized.

According to which type of research is done, the qPCR method can have it advantages or disadvantages over plaque assay. For detecting viral titer and infectivity of MCMV, plaque assay is still a more accurate method because of its ability to detect only infectious viral particles and it cannot be replaced. On the other hand, although qPCR does not differentiate between active or latent virus, specificity and sensitivity of qPCR is important in research on efficiency of newly developed drugs for infection treatment because it shows us the information of the effectiveness of the viral DNA synthesis under certain conditions.

Some future perspective for this area of research could be implementation of different cell types that are known to carry latent CMV to detect differences in viral loads using qPCR and plaque assay or use of advanced cell culture models that replicate the latent and lytic phases of CMV infection. This presents novel experiments for better understanding of the conditions that cause viral reactivation from latency. Similarly, detection of different markers which would differentiate active and non-active viral particles would help into better quantification with qPCR and potentially eliminate the need for plaque assay completely, therefore speed up many studies on CMV infection in general.

6. Conclusion

Accurate and specific methods for detecting newly synthesized MCMV virions in cells and tissues are crucial in detecting potential antiviral mechanisms for infection treatment and drug discovery. While the standard approach in most laboratories involves quantifying infectious MCMV through plaque assays, this method has some limitations when it comes to accurately detecting the virus. In our comparison of plaque assays and quantitative PCR as second method, qPCR showed significantly higher average PFU/mL compared to plaque assays. The main reason behind this dissimilarity is the ability of qPCR to detect both infectious and non-infectious viral particles. By targeting specific genomic sequences, qPCR can identify viral particles even in their latent and non-active states, therefore providing a more complete picture of viral presence.

However, it is important to recognize that plaque assays have a critical role in measuring viral titer and infectivity. Plaque assays, therefore, continue to be necessary in studies requiring precise quantification of infectious virions. On the other hand, qPCR is attractive for its ability to be very precise and sensitive. While it does not recognize the difference between active and latent viruses, it can give insight of the efficiency of viral DNA synthesis under specific conditions, making it a key tool in the research of advanced infection treatments.

In conclusion, the choice between plaque assays and qPCR depends on the specific purposes of the research. Plaque assay remains the first choice in measuring infectivity, while qPCR's sensitivity and precision are important in evaluating the effectiveness of antiviral treatments. Future research in discovering different innovative methods in detection and recognition of CMV virus in different phases in infection would help in deeper understanding of CMV infection and altogether herpesvirus infections.

7. Literature

1. McGeoch DJ, Rixon FJ, Davison AJ. Topics in herpesvirus genomics and evolution. Virus Res. 2006 Apr;117(1):90–104.
2. Ryu WS. Herpesviruses. In: Molecular Virology of Human Pathogenic Viruses. Elsevier; 2017. p. 125–39.
3. Spear PG, Longnecker R. Herpesvirus Entry: an Update. J Virol [Internet]. 2003 Oct [cited 2023 Apr 11];77(19):10179–85. Available from: https://journals.asm.org/journal/jvi
4. Nguyen C, Kamil J. Pathogen at the Gates: Human Cytomegalovirus Entry and Cell Tropism. Viruses. 2018 Dec 11;10(12):704.
5. Brunson JL, Khoretonenko M V., Stokes KY. Herpesviruses. In: Vascular Responses to Pathogens. Elsevier; 2016. p. 123–36.
6. Sinzger C, Digel M, Jahn G. Cytomegalovirus Cell Tropism. In 2008. p. 63–83.
7. Landolfo S, Gariglio M, Gribaudo G, Lembo D. The human cytomegalovirus. Pharmacol Ther. 2003 Jun;98(3):269–97.
8. Rawlinson WD, Farrell HE, Barrell BG. Analysis of the complete DNA sequence of murine cytomegalovirus. J Virol. 1996 Dec;70(12):8833–49.
9. Mahmutefendić Lučin H, Lukanović Jurić S, Marcelić M, Štimac I, Viduka I, Blagojević Zagorac G, et al. Membraneless Compartmentalization of Nuclear Assembly Sites during Murine Cytomegalovirus Infection. Viruses. 2023 Mar 16;15(3):766.

10. Varnum SM, Streblow DN, Monroe ME, Smith P, Auberry KJ, Paša-Tolić L, et al. Identification of Proteins in Human Cytomegalovirus (HCMV) Particles: the HCMV Proteome. J Virol. 2004 Oct 15;78(20):10960–6.

11. Schleiss MR. Cytomegalovirus. In: Maternal Immunization. Elsevier; 2020. p. 253–88.

12. Vanarsdall AL, Johnson DC. Human cytomegalovirus entry into cells. Curr Opin Virol. 2012 Feb;2(1):37–42.

13. Connolly SA, Jardetzky TS, Longnecker R. The structural basis of herpesvirus entry. Nat Rev Microbiol. 2021 Feb 21;19(2):110–21.

14. Griffiths P, Baraniak I, Reeves M. The pathogenesis of human cytomegalovirus. Journal of Pathology J Pathol [Internet]. 2015 [cited 2023 Apr 12];235:288–97. Available from: https://pathsocjournals.onlinelibrary.wiley.com/doi/10.1002/path.4437

15. Jean Beltran PM, Cristea IM. The life cycle and pathogenesis of human cytomegalovirus infection: lessons from proteomics. Expert Rev Proteomics. 2014 Dec 18;11(6):697–711.

16. Lučin P, Kareluša L, Blagojević Zagorac G, Mahmutefendić Lučin H, Pavišić V, Jug Vučko N, et al. Cytomegaloviruses Exploit Recycling Rab Proteins in the Sequential Establishment of the Assembly Compartment. Front Cell Dev Biol. 2018 Dec 4;6.

17. Gibson W. Structure and Formation of the Cytomegalovirus Virion. In 2008. p. 187–204.

18. Close WL, Anderson AN, Pellett PE. Betaherpesvirus Virion Assembly and Egress. In 2018. p. 167–207.

19. Das S, Ortiz DA, Gurczynski SJ, Khan F, Pellett PE. Identification of Human Cytomegalovirus Genes Important for Biogenesis of the

Cytoplasmic Virion Assembly Complex. J Virol [Internet]. 2014 Aug 15 [cited 2023 Apr 19];88(16):9086. Available from: /pmc/articles/PMC4136295/

20. Lučin P, Jug Vučko N, Karleuša L, Mahmutefendić Lučin H, Blagojević Zagorac G, Lisnić B, et al. Cytomegalovirus Generates Assembly Compartment in the Early Phase of Infection by Perturbation of Host-Cell Factors Recruitment at the Early Endosome/Endosomal Recycling Compartment/Trans-Golgi Interface. Front Cell Dev Biol. 2020 Sep 11;8.

21. Homman-Loudiyi M, Hultenby K, Britt W, Söderberg-Nauclér C. Envelopment of Human Cytomegalovirus Occurs by Budding into Golgi-Derived Vacuole Compartments Positive for gB, Rab 3, Trans-Golgi Network 46, and Mannosidase II. J Virol. 2003 Mar;77(5):3191–203.

22. Cepeda V, Fraile-Ramos A. A role for the SNARE protein syntaxin 3 in human cytomegalovirus morphogenesis. Cell Microbiol. 2011 Jun;13(6):846–58.

23. Ryu WS. Diagnosis and Methods. Molecular Virology of Human Pathogenic Viruses. 2017 Jan 1;47–62.

24. Hudson JB, Misra V, Mosmann TR. Properties of the multicapsid virions of murine cytomegalovirus. Virology. 1976 Jul;72(1):224–34.

25. Dymond JS. Explanatory Chapter. In 2013. p. 279–89.

26. Kamimura Y, Lanier LL. Rapid and sequential quantitation of salivary gland-associated mouse cytomegalovirus in oral lavage. J Virol Methods. 2014 Sep;205:53–6.

27. Payne S. Methods to Study Viruses. In: Viruses. Elsevier; 2017. p. 37–52.

28. Vliegen I, Herngreen S, Grauls G, Bruggeman C, Stassen F. Improved detection and quantification of mouse cytomegalovirus by real-time PCR. Virus Res. 2003 Dec;98(1):17–25.

Ema Belužić

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Home: Čakovec (Croatia)

WORK EXPERIENCE

Operator in pharmaceutical production
JADRAN – GALENSKI LABORATORIJ d.d. [03/2023 – 07/2023]

City: Rijeka
Country: Croatia

Operator in pharmaceutical production. Performing product process controls.

Scientific curator, internship
Cell Networks GmbH [05/2022 – Current]

City: Heidelberg
Country: Germany

Scientific data curation form online databases (PubMed).

Microbiology lab assistant, internship
BIOINSTITUT d.o.o. [07/2020 – 08/2020]

City: Čakovec
Country: Croatia

Culturing, examination, and identification of microorganisms, mainly bacteria, in food.

EDUCATION AND TRAINING

Masters program Biotechnology in medicine
University of Rijeka, Department of Biotechnology [01/10/2021 – Current]

Address: 51000 Rijeka (Croatia)
Website: https://www.biotech.uniri.hr/hr/

Bachelor's degree in Biotechnology and drug research
University of Rijeka, Department of Biotechnology [01/10/2018 – 01/09/2021]

Address: 51000 Rijeka (Croatia)
Website: https://www.biotech.uniri.hr/hr/
Thesis: The role of DTNBP1/dysbindin-1 in schizophrenia, Mentor: doc.dr.sc. Nicholas J. Bradshaw

Gymnasium diploma
Linguistic gymnasium, Gymnasium Josip Slavenski, Čakovec [01/09/2014 – 15/06/2018]

Address: 40000 Čakovec (Croatia)
Website: https://gimnazija-cakovec.hr/

LANGUAGE SKILLS

Mother tongue(s): **Croatian**

Other language(s):

English

LISTENING C1 **READING** C1 **WRITING** C1

SPOKEN PRODUCTION C1 **SPOKEN INTERACTION** C1

German

LISTENING B1 **READING** B1 **WRITING** B1

SPOKEN PRODUCTION B1 **SPOKEN INTERACTION** B1

French

LISTENING A1 **READING** A1 **WRITING** A1

SPOKEN PRODUCTION A1 **SPOKEN INTERACTION** A1

Levels: A1 and A2: Basic user; B1 and B2: Independent user; C1 and C2: Proficient user

DIGITAL SKILLS

MS Office (MS Word, MS Powerpoint, MS Excel, MS Publisher, MS Outlook, MS Access, MS Teams) / Molecular design software (PyMol, Chimera, Avogadro) / R / R Studio / R markdown / Online Tool for Bioinformatics (BLAST, GeneCards, Gene Ontology, KEGG, etc.) / Social media/ Social networks

CONFERENCES AND SEMINARS

Future and perspective

[Department of Biotechnology, Rijeka, 10/2021]

Conference intended for all students in the biomedical field, and its goal is to bring the students closer to various employment and career development opportunities after completing their studies.

VOLUNTEERING

Project "Student mentor"

[University of Rijeka, Department of Biotechnology, 01/10/2019 – Current]

Assistance to new students in the Department of Biotechnology in finding their way and adjusting to their studies.

Organization "Zora"

[Čakovec, 01/09/2015]

A non-profit organization committed to preserving human rights and equality and developing and changing the community.

POPULARIZATION OF SCIENCE

Traveling scientists, University of Rijeka

[01/10/2019 – Current]

A project that encourages the education of younger generations in the basics of natural sciences by demonstrating simple and interesting experiments.

Open days of Department of Biotechnology

[2022]

Presentation of projects and work that happens in the department to future students.