A Reverse Transcription-Quantitative Polymerase Chain Reaction-based Microneutralization Assay for Assessing Human Cytomegalovirus-Neutralizing Antibody Activity

by

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Abstract

Human cytomegalovirus (HCMV) is a leading cause of sensorineural hearing loss and neurodevelopmental delays resulting from congenital infections and a major reason for morbidity and mortality in immunocompromised patients. To prevent these complications of HCMV infection, developing a prophylactic vaccine with high efficacy has been considered a top priority to public health. Neutralization assays are fundamental for evaluating neutralizing activities of antibodies in vaccine development, but traditional immunostaining-based neutralization assays are tedious and laborious, making it less suitable for screening large numbers of samples. A reverse transcription-quantitative polymerase chain reaction (RT-qPCR) -based microneutralization assay targeting the immediate-early gene transcript of HCMV was previously reported as an alternative solution for testing neutralizing activity in a timely manner. In a separate study, a cell-lysate-generation method was capable of simplifying RNA isolation steps for RT-qPCR analysis and reducing running costs, but both methods have not been well validated for clinical application.

In this study, both methods were combined and evaluated with a laboratory-adapted HCMV clinical strain VR1814 with wide tropism in fibroblasts (MRC-5), epithelial cells (ARPE-19) and endothelial cells (HMEC-1). My goal was to assess neutralizing activities of human immunoglobulins (HIG) and monoclonal antibodies on clinical HCMV strains by comparing my combined RT-qPCR-based neutralization assay with immunostaining.

My RT-qPCR assay had a sensitivity of 0.6 infectious units (IU)/reaction with a linear range from 10^4 to 1 IU/reaction. The assessment can be conducted as early as 20 h post-infection. High agreement was observed between the results of RT-qPCR and immunostaining assays in determination of antibody neutralization against VR1814

and clinical HCMV strains. In contrast to the laboratory-adapted strain VR1814, neutralization resistance to immunoglobulins and monoclonal antibodies was observed in HCMV in primary clinical samples of urine, milk and saliva.
Thus, my RT-qPCR assay is a useful alternative method of assessing HCMV-neutralizing activity with higher accuracy and precision than the immunostaining assay. The neutralization resistance of HCMV from clinical specimens suggests that current monoclonal antibodies may be incapable of preventing CMV replication *in vitro*.

Preface

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Cytomegalovirus (CMV) vaccine development: a study of viral tropism, genomic diversity and neutralizing epitopes in CMV positive clinical samples", No.Pro00074348, July 31, 2018.

No part of this thesis has been previously published.

Dedication

To my beloved parents, my pillars of strength, thank you for all your love and support.

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List of Abbreviations

ATCC	American type culture collection
BI ELISA	Binding inhibition ELISA
BKV	BK virus
CL buffer	Cell lysis buffer
CL	Cell-lysis
CV	Coefficient of variation
cDNA	Complementary DNA
Ct	cycle threshold
CMV	Cytomegalovirus
CPE	Cytopathogenic effect
DMEM	Dulbecco's modified eagle’s medium
ELISA	Enzyme-linked immunosorbent assays
EGF	Epidermal growth factor
EBV	Epstein-Barr virus
EBV	Epstein–Barr virus
FBS	Fetal bovine serum
gH	Glycoprotein H
gL	Glycoprotein L
gO	Glycoprotein O
IC50	Half maximal inhibitory concentration
HSV	Herpes simplex virus
h.p.i.	Hours post-infection
HCMV	Human cytomegalovirus
HHV-6	Human herpesvirus 6
HIG	Human immunoglobulin
IE	Immediate-early
IgG	Immunoglobulin G
IU	Infectious units
LOD	Limit of detection
Tm	Melting temperature
MEM	Minimum essential medium eagle
NK	Natural killer
PBS	Phosphate-buffered saline
PFU	Plaque-forming unit
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
rcf	relative centrifugal force
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SD	Standard deviation
IFNαβ	Type I interferons
UL	Unique long

US	Unique short
VZV	Varicella zoster virus

Chapter 1 Literature Review

1.1 History

In 1881, German scientist Hugo Ribbert observed “protozoan like” cells in the kidney of a stillborn, which was believed to be the first discovery of cytomegalovirus infections (1). Seven decades later, Smith, Rowe *et al*, and Weller *et al* independently isolated a new virus from different organs of patients in 1956 and 1957, and found the virus was so specific to humans that it could not be cultured in animal models (2). In 1960, Weller named this virus as “cytomegalovirus (CMV)”, referring to the characteristic enlargement of cells with viral inclusion bodies caused by CMV infection in pathology samples (3).

1.2 Classification

Cytomegalovirus is a herpesvirus, belonging to the Betaherpesvirinae subfamily (4). Other well-known herpesviruses that are highly endemic among humans include herpes simplex virus (HSV) 1 and 2, varicella zoster virus (VZV), Epstein–Barr virus (EBV), human herpesvirus 6 (HHV-6) and HHV-7 (4–6).

1.3 Structure

HCMV is a linear double-stranded DNA virus and one of the largest viruses in the world (7). The genome of HCMV is around 235kb in size, containing unique long (UL) and unique short (US) regions, with terminal and internal repeated sequences flanking each end of the region (8). The genome is enclosed within a 130 nm diameter nucleocapsid (9). Outside the capsid, there is a protein layer named the tegument, which contains virus-encoded factors essential for transcription initiation and viral immune evasion (10). The tegument itself is surrounded by an envelope with more than 20 virus-encoded glycoproteins, some of which congregate to form complexes, such as the glycoprotein H (gH)/gL/gO complex (11,12). Figure 1.1 demonstrates the structure of HCMV (13).

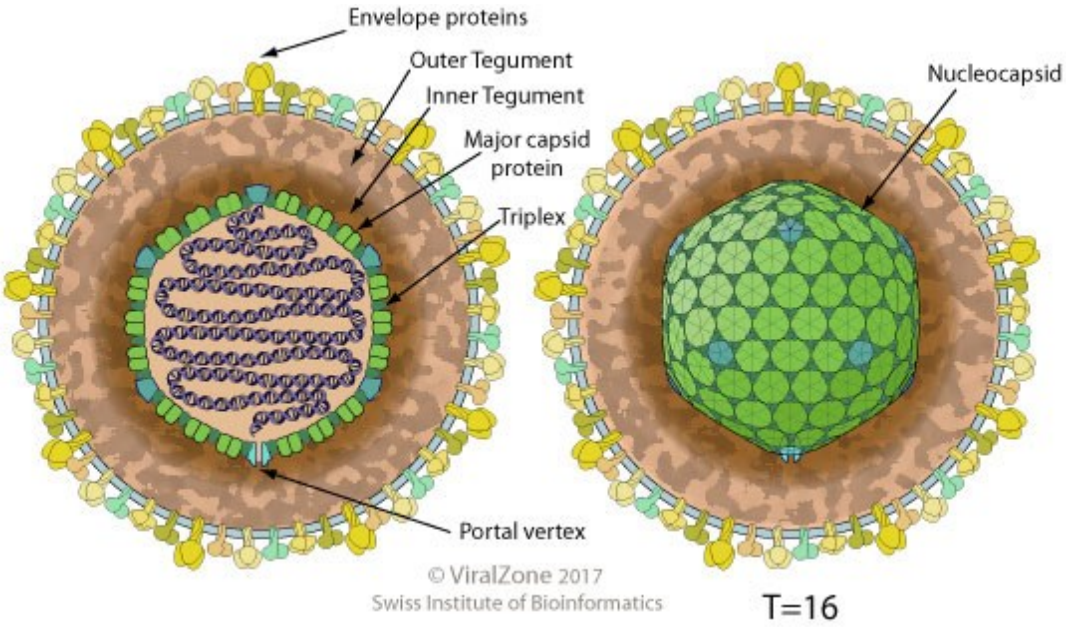


Figure 1.1 The structure of human cytomegalovirus. Reprinted with the permission from Swiss Institute of Bioinformatics (CC BY-NC-ND 4.0) (13).

1.4 Replication of cytomegalovirus

HCMV can replicate in a wide range of organs and tissues in the human body (14). To initiate entry, multiple glycoproteins in the virion envelope are involved in adherence to cell surface receptors and fusion with cellular membranes, but the role of each glycoprotein in viral entry is not well understood (15,16). gB is necessary for membrane fusion (17). Some studies suggested mutants lacking gB were unable to enter cells unless they were treated with polyethylene glycol (PEG), a chemical fusogen, or the entered cells were capable of expressing gB (17,18). gH/gL is crucial for HCMV binding to cellular receptors, as many studies suggested that a lack of gH or gL led to the loss of viral entry capacity (17,18). Another function of gH/gL is to aggregate with other glycoproteins to assemble glycoprotein complex, such as the trimeric complex gH/gL/gO and pentameric complex gH/gL/UL128-131; the latter is indispensable for HCMV to enter epithelial and endothelial cells (15,19).

After virions enter cells and the genomes are delivered to the cellular nucleus, gene expression starts (20). Genes of HCMV can be sorted into three categories according to the time of their expression: immediate-early (IE) (α) genes, early (β) genes, and late (γ) genes (1).

IE genes, most of which are located in the UL122 to UL123 gene regions (21), are the first HCMV genes responsible for starting transcription after infection (22). IE

genes include 2 regulatory products, immediate-early protein 1 (IE1) and 2 (IE2), both of which are involved in immune evasion activity and optimizing activation of the early gene (23).

The expression of HCMV early genes is activated by IE proteins and requires cellular proteins to complete their transcription (20). The presence of early genes can be detected as early as 6 hours post-infection (h.p.i.) depending on viral strains (24). Products of early genes play a key role in regulating viral DNA synthesis and modulating host cell environment for replication (20).

HCMV late genes are initiated after viral gene replication and expressed after 24 h.p.i. (25). Compared with IE and early genes, there are fewer late genes that have been less well studied. The main function of late gene products is viral assembly and maturation along with early gene products (26).

Once viral assembly has been completed, enveloped virions are delivered to the cellular surface and released (27). The entire HCMV replication cycle takes around 72 hours to complete and is strain-dependent (28). The figure 1.2 shows an overview of the HCMV life cycle (29).

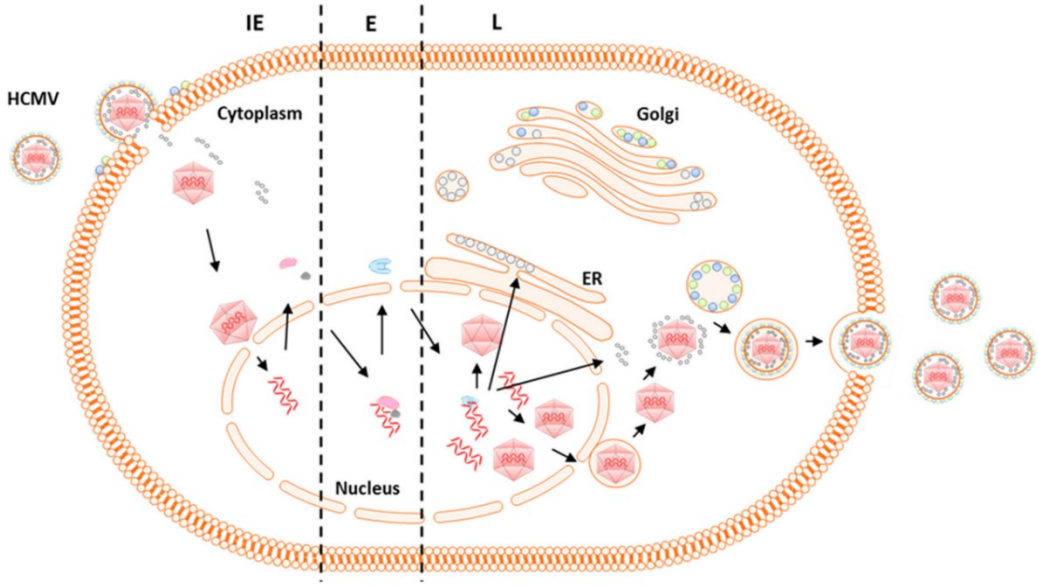


Figure 1.2 Overview of the HCMV life cycle
Human cytomegalovirus (HCMV) life cycle. HCMV enters the cell through interaction of the host receptors with specific viral glycoproteins. Capsid and tegument proteins are release into the host cytosol. The capsid releases the viral genome into the nucleus, leading to the expression of immediate early (IE) genes. The IE proteins activate the expression of the early (E) genes. The E proteins initiate viral genome replication and the expression of late (L) genes. The L gene expression initiates the capsid assembly and the expression of tegument- and glycoproteins. The genome-loaded capsid enters the cytosol via nuclear egress. The capsid associates with the tegument proteins. The capsid acquires the viral envelope by budding into intracellular vesicles. The enveloped viral particles are released into the extracellular space. Reprinted with the permission from *International Journal of Molecular Sciences* (CC BY 4.0) (29).

1.5 Viral strains and cell tropism

HCMV can replicate in a wide range of cell types *in vivo*. The strains isolated from clinical specimens are known as wild-type strains or clinical strains. However, it is difficult to produce a high yield of HCMV in cell lines other than fibroblasts by using clinical strains (30). After several passages in fibroblasts, high titers of HCMV can be achieved, but one disadvantage is that mutations will also be quickly induced during this process. Those mutated strains are refer to laboratory-adapted strains(31).

For decades, laboratory-adapted strains have been widely used in vaccine development and neutralizing activity assessment (32). Well-known laboratory-adapted strains include AD169 and Towne; these strains can quickly generate a large number of infectious virions required to meet research needs (33). However, many genes in laboratory-adapted strains unnecessary for growth in fibroblasts have been mutated or completely lost (34,35). Over 100 mutations can be found in AD169 and Towne (36), including mutations in HCMV genes RL13 and UL128, leading to the loss of the ability of these strains to replicate in other differentiated cell types, such as epithelial and endothelial cells (35,37,38). Even for clinical isolates propagated in fibroblasts for only a limited number of passages, mutations can be induced, meaning tropism for non-fibroblast cells is gradually lost (39).

Mutations in laboratory-adapted strains result not only in the change of cell tropism but also in the mechanism of transmission. HCMV virions mainly spread to adjacent cells *in vivo* (40,41). Little is known about the function and mechanism of this cell-to-cell transfer, but some studies have suggested that this transmission significantly increases the ability of HCMV to resist neutralizing antibodies, which might contribute to immune evasion and latency development in hosts (42,43). However, virions produced by laboratory-adapted strains include cell-free viruses. In this setting, infection of new cells results from both cell-to-cell transfer and infection by cell-free viruses that are more sensitive to neutralizing activity (42). Based on the cell tropism of HCMV, studies using human fibroblasts alone are not a good surrogate for simulating HCMV neutralizing activity *in vivo*.

1.6 Pathogenesis of cytomegalovirus infection

1.6.1 Epidemiology

HCMV infection is very common worldwide, with a seroprevalence of 35 - 80%

in adults in industrialized countries increasing to almost 100% in developing countries (44). In the United States, a nationwide study showed that 58.9% of the population over 6 years old had been infected by HCMV, and the seroprevalence increased with age to 90.8% in age groups over 80 (45). In Canada, a cohort study conducted in Quebec suggested that 40.4% of women of child-bearing age were HCMV seropositive (46). A local study done in Edmonton investigated mothers with very low birth weight infants, 55% of who were HCMV positive (47).

1.6.2 Transmission

HCMV can spread through both horizontal and vertical transmission (48).

The most common transmission of CMV is horizontal transmission, which usually requires direct contact with body fluids (e.g. urine, saliva or semen) that contain infectious particles (49). HCMV rarely causes symptoms in healthy hosts. Most infected people are unaware of the infection. However, immunocompromised patients are susceptible to greater HCMV-associated morbidity (50). HCMV is the most common pathogen responsible for viral opportunistic infections and the major cause of morbidity and mortality in immunocompromised patients (51). HCMV can also be transmitted through blood transfusion or transplantation of organs from seropositive donors. Transplant recipients at the highest risk were those who were HCMV seronegative before transplant but received organs from HCMV seropositive donors (HCMV D+/R-) (52).

In addition to horizontal transmission, CMV is unique among herpesviruses in that it can also be commonly transmitted from mother to child in three ways: transplacental, intrapartum and via breast milk (53). Among the three transmission routes, severe consequences usually are limited to infants whose mothers experience primary infection with HCMV during pregnancy (54). Congenital HCMV infection leads to sensorineural hearing loss and neurological impairments (55). HCMV infection is the most common congenital infections in Canada, with an estimated 0.42 - 0.45% of infants infected (54,56).

1.6.3 Immune response and latency of cytomegaloviruses

The mucosal epithelium is the first barrier of defense from infection, so epithelial cells are most likely to be major cell targets used by HCMV to enter a new host (57).

After the primary infection, both innate and adaptive immune responses are elicited (58). Natural killer (NK) cells and type I interferons (IFNαβ) are crucial for innate response to viral replication and are released within 6 hours after primary infection (59,60). The adaptive response to HCMV involves humoral and cellular immunity (49). T cell mediated response plays a key role in this process and dominates in the peripheral blood of hosts (61). Multiple antibodies specific for HCMV are elicited in the second stage of immune response and contribute to the ultimate control of viral infection (49). However, endothelial cells help the spread of HCMV *in vivo* and facilitate HCMV dissemination to myeloid lineage cells (62). Like all herpesviruses, HCMV will ultimately reach a balance with the immune system and evade immune surveillance (63). Finally, the virus will use myeloid lineage as a reservoir to establish persistent latency (49).

During latency, HCMV can reactivate periodically resulting in asymptomatic shedding of infectious virions in urine, saliva, semen, and cervical secretions (49). However, when immunity is suppressed, reactivation and reinfection of HCMV will occur frequently and can cause symptoms (55). HCMV can also be transiently reactivated during pregnancy and shed in breast milk, which is the most common route for infection of breastfed newborns (64).

1.7 Vaccine development and neutralizing antibody

The serious consequences of HCMV infection are a wide public health concern. The development of a prophylactic or therapeutic vaccine to prevent worldwide HCMV transmission has been identified as a priority by the Institute of Medicine of the United States and the Government of Canada (65,66).

Currently, there is no licensed vaccine available, but several HCMV vaccine candidates have been developed in the past decades (67). A gB/MF59 vaccine candidate developed based on the HCMV strain Towne was shown to have an efficacy of 50% (68), which is the highest efficacy of all vaccine candidates in phase II clinical trials (69). However, 50% efficacy is still lower than the required efficacy expected by public health. In addition to gB, gH and UL128-131 pentamer complex are common alternate immunogen choices for vaccine development as gH plays an indispensable role in viral fusion and the pentameric complex is necessary for entry into epithelial and endothelial cells (70,71). However, most vaccine candidates were developed

based on laboratory-adapted strains and clinical isolates passaged in fibroblasts that represent true wild-type strains poorly due to induced mutations (72).

1.7.1 HCMV neutralization resistance in primary urine samples

A recent study reported by Cui *et al*. suggested that wild-type HCMV in primary urine samples may be resistant to neutralizing antibodies (43). In their study, HCMV in urine samples from 10 congenitally infected newborns demonstrated a profound neutralization resistance against monoclonal antibodies specific for gB, gH/gL, and the pentameric complex of HCMV. This antibody resistance could be quickly lost after cultivation in fibroblasts for just one passage. Their study further confirmed the importance of investigating HCMV neutralization in both fibroblast and non-fibroblast cells, and partially explained why antibodies with high neutralizing activities *in vitro* were less effective *in vivo*.

1.8 Laboratory techniques for HCMV detection

After HCMV was isolated and named in 1960, a multitude of methods have been applied to identify HCMV infection and quantify HCMV viral load (73).

Culture-based methods were commonly used for HCMV detection and diagnostics in the clinical setting until molecular technology launched recently, and are still an essential technique for research in many aspects such as immunology and vaccine development.

The most traditional and historically used method to determine HCMV viral quantity is the plaque assay, the first publication of which for HCMV quantification dates back to 1952 (74). A variety of sample types (e.g. urine, blood leukocytes or bronchoalveolar lung lavage fluid) can be used in a plaque assay. The turnaround time of the plaque assay was seven days for laboratory-adapted HCMV strains and more than two weeks for clinical strains in fibroblasts (75,76). The plaque assay is still a common method of determining titers of viral stock in research and crucial for viral titration studies, but the long processing time and high variability make it less applicable to studies with a large sample size (77).

Shell vial assay is another culture-based assay. By counting the fluorescence-labelled foci formed by monoclonal antibodies against immediate-early HCMV antigens, shell vial assay could shorten the processing time to one or two days and be

more quantitively sensitive than plaque assays (78). However, to maximize its sensitivity, more than 4×10^6 leukocytes were recommended to use for HCMV quantification, which is less applicable in many settings (79).

Antigenemia assay is a common method for diagnosis of HCMV infection in transplant patients, which directly stains blood leukocytes with monoclonal antibodies targeting protein pp65 encoded by UL83 and therefore eliminates the need for viral culture (80). Antigenemia assay is a rapid method with high sensitivity, but it is less useful in this thesis as it can only process peripheral blood samples (81).

Immunostaining assay is a classic method in pathological diagnosis. Novel microimmunostaining assays have been developed to detect the signal of infected cells by binding labelled antibodies to specific HCMV antigens (82,83). The addition of secondary antibodies can further amplify signals and reduce background caused by unspecific binding. A colour-producing reaction catalyzed by enzyme makes stained proteins countable under microscopes (84). Microimmunostaining assay can simplify the tedious procedure of traditional culture-based methods and shorten incubation time, thereby accelerating HCMV quantification (85).

1.8.1 Quantitative PCR

The development of molecular techniques fundamentally changed the way of HCMV detection (86). PCR, which was invented by Kary B. Mullis in 1983, is one of the most revolutionary technologies in the 20th century. PCR can produce numerous copies of a DNA segment, making it possible to detect minute amounts of specific DNA fragments from samples, thereby significantly increasing sensitivity and specificity (87).

The earliest PCR was conventional PCR, which runs DNA samples with a set of DNA fragments of known-size (known as a PCR ladder) on an agarose gel simultaneously and therefore determines the presence and size of DNA products (88). The drawbacks of conventional PCR include: (1) it is a qualitative method; (2) the procedure is labour-intensive and time-consuming (3 - 4h); (3) carry-over contamination may be caused in the post-PCR step. Those drawbacks prevent conventional PCR from being used for HCMV diagnosis, but it is still a powerful tool for isolation or cloning purposes (89).

Quantitative PCR (qPCR) is a well-developed PCR method that can provide

quantitive information during amplification. The closed tube operation of qPCR significantly reduces carry-over contamination; the whole process only takes a few hours; qPCR can be sequence-specific with an application of a probe system. As HCMV is a double-stranded DNA virus, qPCR is capable of quantifying HCMV DNA fragments from various specimens in a timely manner and has become the most common HCMV diagnostic method in a clinical setting (90,91).

1.8.2 RT-qPCR

RT-qPCR is a molecular technique that can convert a particular RNA segment into a complementary DNA (cDNA) and then use the cDNA as the template for qPCR in order to quantify the RNA expression of target viruses (92).

RT-qPCR has the same advantages as qPCR including high sensitivity, high reproducibility and cost-effectiveness. For DNA viruses such as HCMV, RT-qPCR is able to detect mRNA fragments, thereby indicating the presence of viral replication (93). Due to this feature of RT-qPCR, some RT-qPCR assays targeting late gene transcripts have been developed between 2000 to 2010 aiming to accurately monitor HCMV replication in transplant recipients (94,95). However, those assays had a relatively low clinical sensitivity and failed to demonstrate superiority over qPCR assays to indicate of HCMV replication *in vivo*, so the attempt of using RT-qPCR for diagnostics was discontinued.

Nevertheless, RT-qPCR still has been used as an effective indicator of viral infectivity in neutralization studies (96). Most of the RT-qPCR-based neutralization assays were developed targeting IE gene transcripts with a few targeting late gene transcripts (97,98). In an HCMV neutralization study, compared with plaque assays that can only count 5 to 100 plaques per well (74,77), the dynamic range of RT-qPCR can be as wide as three orders of magnitude, which is a significant advantage compared to traditional methods (99).

1.9 Neutralization assays for HCMV

To facilitate HCMV vaccine development, it is important to evaluate the neutralizing activity of monoclonal antibodies against wild-type HCMV strains. A neutralization assay is a test to determine neutralizing antibody activity capable of blocking viral infectivity (100). In a neutralization test, serial dilutions of antibodies

are mixed with an equal volume of HCMV samples and incubated for 1-4 hours. After incubation, the mixtures are used to inoculate cell monolayers. The proportion of HCMV virions being neutralized can be calculated by comparing the level of viral infectivity in the presence and absence of antibodies in cell culture, and therefore the neutralizing ability of antibodies can be measured and compared (101). The concentration of antibody that can inhibit 50% of viral infectivity is called the half maximal inhibitory concentration (IC50), which is an important value for neutralizing antibody evaluation and comparison (102).

A variety of HCMV detection methods have been applied to determine viral infectivity in cell monolayers in order to calculate IC50 values in neutralization assays, but a gold standard of neutralization method with both accuracy and efficiency is still lacking.

1.9.1 Plaque reduction neutralization assay

Plaque assay is the most traditional culture-based method to detect infectious HCMV virions, thereby being the original method applicable to neutralization study (101). After viral culture, monolayers are stained with crystal violet to count plaques formed by viral propagation in order to generate neutralization curves and calculate IC50 values (101). However, the experimental procedure of plaque assay is time-consuming and labour-intensive (75). A collaboration study among twelve laboratories demonstrated that the high variability and low agreement of plaque reduction neutralization assays for clinical isolates and the challenge of determining formed plaques among different individuals (75). However, as the most traditional visualized neutralization test, plaque reduction neutralization assay is still widely used in many laboratories.

1.9.2 Immunostaining-based neutralization assay

Immunostaining is another visualized method able to detect viral infectivity and widely used as a rapid alternative to plaque assays in neutralization studies.

Immunostaining can be divided into immunoperoxidase and immunofluorescence staining, both of which are applicable to neutralization assays (85). The latter is more common due to its clearer signals, fewer false positives and higher sensitivity than the former (83,103). Regardless of problems with nonspecific staining and backgrounds

caused by endogenous peroxidase, the biggest advantage of immunoperoxidase is that there is no specialized equipment (e.g. fluorescence microscopes) required and the quantification procedure can be completed by an ELISPOT analyzer rather than manual counting under a fluorescence microscope (104). Immunoperoxidase staining-based neutralization assays have a simplified experimental procedure compared to the plaque reduction neutralization assay and avoid the confusion of determination of plaques, but low reproducibility (around 12% of CV), the need for immediate processing and the subjective and tedious counting step still hindered it from being the gold standard of neutralization assays (82).

1.9.3 RT-qPCR-based neutralization assay

RT-qPCR is able to measure gene transcription for DNA viruses, which can be considered as an indicator of viral infectivity and used in neutralization assays.

1.9.3.1 Nucleic acid extraction for RT-qPCR-based neutralization assay

For RT-qPCR, nucleic acid extraction is the primary step for downstream molecular detections. The first DNA purification dates back to 1869, when Friedrich Miescher, a Swiss physician, first obtained purified leucocytes using surgical bandages (105). Over the years, a variety of extraction methods have been developed and various commercial offerings are available on the market. The advantages of nucleic acid extraction for downstream detection include contamination reduction, nucleic acid concentration, and RNA or DNA isolation (106). However, nucleic acid extraction also causes loss of material, requires special instruments for large-scale samples, and consumes time as well as labour to perform (107).

Commercial cell lysis buffer (CL buffer) is another option to generate PCR or RT-PCR-ready cell lysates capable of direct quantification and therefore avoid nucleic acid extraction steps (108), but many clinical specimens contain potential PCR inhibitors (e.g. heme in blood or urea in urine) that are difficult to eliminate only by CL buffer (109). The high cost of commercial CL buffer is another disadvantage that has hindered its being used for neutralization tests (110,111).

An in-house CL buffer generation method described by Shatzkes *et al*. was able to prepare RT-qPCR-ready cell lysates without RNA extraction for RT-qPCR analysis and has been successfully applied to an RT-qPCR-based neutralization assay (112).

The in-house CL buffer can be an inexpensive alternative to commercial CL buffer, but whether it can prevent PCR inhibition caused by factors in clinical samples and be applied to neutralization assays against wild-type HCMV virions still need to be evaluated.

1.9.3.2 RT-qPCR for neutralization

For DNA viruses, RT-qPCR can quantify the gene expression level and assess the level of viral infectivity. Several publications have developed RT-qPCR-based neutralization assays for DNA viruses as an alternative to traditional plaque reduction neutralization assays (96,97,113). A paper published in 2015 described an RT-qPCR-based microneutralization assay for HCMV (97). The advantages of this method include: (1) application of a one-step RT-qPCR method. A one-step method enables the occurrence of the entire reaction in one single tube, which is more convenient than two-step methods and has less possibility of contamination (114); (2) application of a SYBR green system. SYBR green is a fluorescent dye that binds to double-stranded DNA and therefore indicates DNA amplification, which is less expensive than a primer-probe-based RT-qPCR detection (115); (3) primers spanning an exon-exon boundary. Primers that anneal to exons with an intron insert can amplify cDNA only and therefore avoid potential genomic DNA amplification, which enables skipping the DNase digestion step in RNA purification procedure (105).

This RT-qPCR-based neutralization assay is a rapid method that can be conducted as early as 6 h.p.i. with high automation and sensitivity, but has not been validated and systematically compared with traditional neutralization assays (97).

1.9.4 Other neutralization assays

Other HCMV detection methods are less common to be applied to neutralization studies.

Some studies applied qPCR to indirectly reflect viral infectivity (116–118), which may be less accurate because positive PCR results only indicate the presence of DNA amplicons but fail to differentiate infectious HCMV virions and HCMV DNA fragments. Therefore, free HCMV DNA will cause high variability and affects the accuracy of neutralization results (91).

Indirect and binding inhibition ELISA (BI ELISA) were also used in

neutralization assays (119). In a BI ELISA-based neutralization assay, the antibody-virus mixtures were transferred to a microplate coated with capture antibodies specific to viral types, followed by a regular sandwich ELISA procedure to detect unneutralized viral antigens (120,121). The advantage of BI ELISA-based neutralization assay is avoiding culture steps in neutralization tests, but this simplification leads to an increase in the nonspecific background and low sensitivity (120). Moreover, those ELISA-based neutralization assays were more applicable to laboratory-adapted HCMV strains which release more cell-free viruses. Since wild-type HCMV strains transmit through a cell-to-cell route, ELISA-based methods were less likely to meet the need of this thesis.

Flow cytometry also has been used in some neutralization studies (122,123). A new report of flow cytometry demonstrates its capacity to preserve viral infectivity for further studies after cell sorting (124), which is also superior to endpoint techniques such as immunostaining and plaque assays. However, the processing steps of a flow cytometry-based assay could cause the lysis of infected cells, leading to a bias in the proportion of infected cells to healthy cells (125,126). The subjective gating step and requirement of special equipment are other disadvantages of a flow cytometry-based neutralization assay (124,127). A study compared the flow cytometry-based neutralization assay with a plaque reduction neutralization assay and suggested that flow cytometry did not have obvious advantages in terms of accuracy and precision (128).

Overall, various disadvantages of qPCR, ELISA and flow cytometry prevented them from being widely applied to neutralization assays.

1.10 Hypothesis

An optimized RT-qPCR-based neutralization assay with RT-qPCR-ready cell lysates not requiring RNA extraction is superior to an immunoperoxidase staining assay in sensitivity, precision and specificity for assessing HCMV-neutralizing antibody activity *in vitro*

1.11 Objectives

1. Optimization and validation of an RT-qPCR-based neutralization assay for quantification of infectious HCMV virions.

2. Optimization and validation of an RT-qPCR-ready cell lysate generation method direct for RT-qPCR analysis without RNA extraction.

3. Comparison of the RT-qPCR assay with the immunoperoxidase staining assay in assessing neutralizing antibody activity against laboratory-adapted HCMV strains and wild-type HCMV from clinical specimens.

Chapter 2 Materials and Methods

2.1 Workflow

The RT-qPCR assay previously described by Wang *et al*. was validated in terms of sensitivity, specificity and precision by using purified RNA from a laboratory-adapted HCMV strain VR1814. The best time point for RT-qPCR analysis on both VR1814 and clinical samples in MRC-5, ARPE-19, and HMEC-1 was determined and compared with an in-house qPCR assay.

Vortex and centrifugation were applied to the cell-lysate-generation method by using VR1814 in MRC-5, ARPE-19, and HMEC-1 in order to eliminate amplification inhibition in RT-qPCR and improve HCMV RNA yields. The number of cells that could be processed by the modified cell-lysate-generation method was determined. The HCMV RNA load provided by the modified method was compared with a commercial RNA extraction kit in all three cell types.

The RT-qPCR assay with the modified cell-lysate-generation method was compared with the immunostaining assay in terms of sensitivity, specificity, neutralization curve generation, and IC50 value determination by using VR1814 in MRC-5, ARPE-19, and HMEC-1.

Both RT-qPCR and immunostaining assays were applied to detect the neutralizing activity of human immunoglobulins and monoclonal antibodies against wild-type HCMV in primary clinical samples including urine, saliva, and breast milk in MRC-5, ARPE-19, and HMEC-1. A flow chart of the entire study design is shown in Figure 2.1.

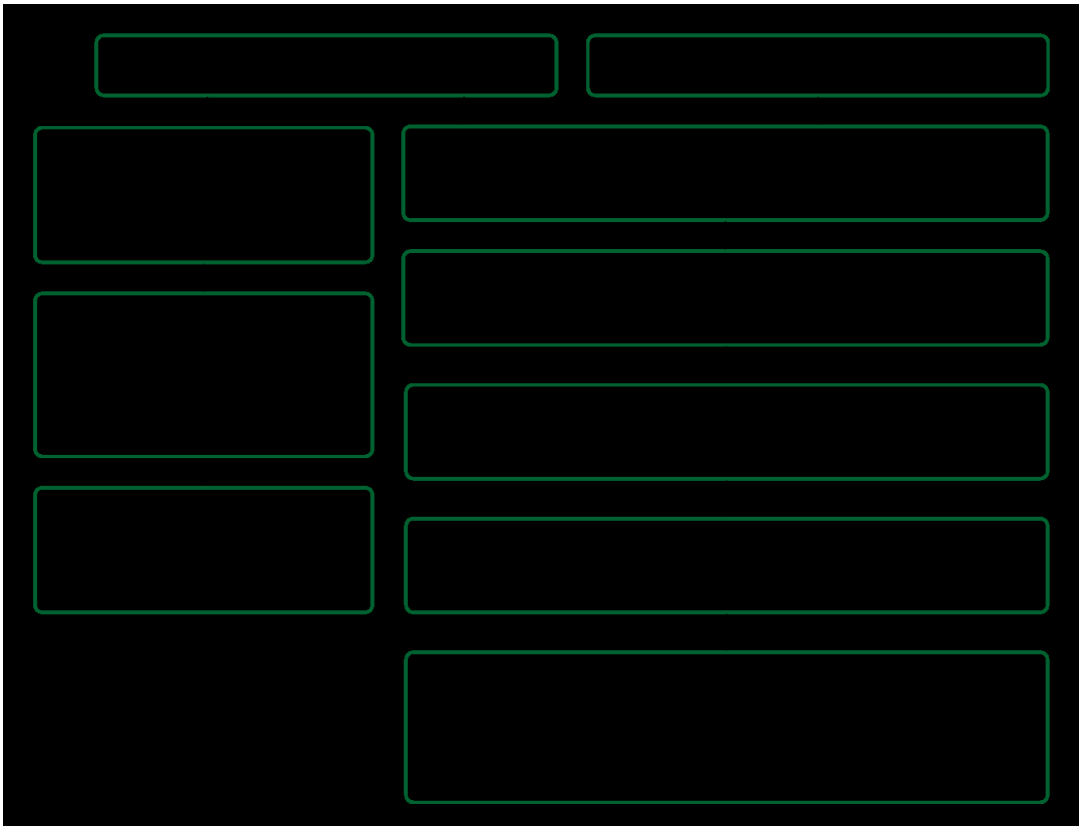


Figure 2.1 Workflow for the study

2.2 Materials

2.2.1 Cells and media

Three cell lines used in this study were obtained from two different sources. The human embryo fibroblasts MRC-5 (CCL-171) and human retinal pigment epithelial cells ARPE-19 (CRL-2302) were obtained from American Type Culture Collection (ATCC; Manassas, US). The human dermal microvascular endothelium cells HMEC-1 (CRL-3243) was kindly provided by Dr. Allan Murray (University of Alberta, Edmonton, Alberta), and had originally been obtained from ATCC.

Every cell line has two modes of medium for cultivation: growth medium that can support cell proliferation and maintenance medium that support cell survival.

The growth medium for MRC-5 contains Minimum Essential Medium Eagle (MEM; Sigma-Aldrich, US) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, US), 2mM L-Glutamine (200mM, Gibco, Thermo Fisher Scientific), and 0.2% Gentamicin (Gibco, Thermo Fisher Scientific). The maintenance medium for MRC-5 contains MEM with 2% FBS, 2mM L-Glutamine, and 0.2% Gentamicin.

The growth medium for ARPE-19 contains Dulbecco's Modified Eagle’s Medium

(DMEM; Sigma-Aldrich) supplemented with 10% FBS, 2.5 mM L-Glutamine. The maintenance medium for ARPE-19 contains DMEM with 2% FBS and 2.5mM L-Glutamine.

The growth medium for HMEC-1 contains MCDB 131 (Sigma-Aldrich) supplemented with 10% FBS, 10ng/ml epidermal growth factor (EGF; 10 ng/ml, Sigma-Aldrich), 10nM L-Glutamine and 1μg/ml Hydrocortisone stock solution (50 μg/ml, Sigma-Aldrich, US). The maintenance medium for HMEC-1 contains MCDB 131 with 2% FBS, 10 ng/ml EGF, 10 nM L-Glutamine and 1μg/ml Hydrocortisone stock solution.

2.2.2 Viruses

Infectious HCMV strain VR1814 (GenBank Sequence Accession: GU179289) originated as a clinical isolate from the cervical secretions of a pregnant woman with a primary HCMV infection was kindly provided by Dr. Elena Percivalle (Policlinico San Matteo Pavia Fondazione IRCCS, Italia).

VR1814 is a laboratory-adapted strain capable of replicating in MRC-5, ARPE-19 and HMEC-1, and can be detected by both the qPCR (described in section 2.3.4) and RT-qPCR (described in section 2.3.6) in this study. Therefore, VR1814 was used as positive controls for viral cultivation, viral replication kinetic generation, neutralization tests, qPCR and RT-qPCR analysis.

2.2.3 Antibodies

Two human immunoglobulins and four monoclonal antibodies were included in this study in order to assess their HCMV-neutralizing activity (Table 2.1). Hizentra (Hizentra®; CSL Behring, King of Prussia, PA) is purified normal immunoglobulin G (IgG) derived from pooled human plasma. Cytogam (CytoGam®; CSL Behring, King of Prussia, PA) is purified IgG directly against HCMV derived from pooled human plasma. Antibody 2B11 (1.5 mg/ml) and antibody 6B4 (5.6 mg/ml) are human monoclonal antibodies against HCMV specifically binding to gB. Antibody 11B12 (1.1 mg/ml) is a human monoclonal antibody against HCMV specifically binding to gH. Antibody 8I21 (8.1 mg/ml) is a human monoclonal antibody against HCMV binding to the HCMV pentamer gH/gL/UL128/130. All the immunoglobulins and antibodies were kindly provided by Pfizer Inc. and stored at 4°C until use.

Table 2.1 Information of antibodies used in HCMV neutralization tests

Name	Antibody types	Specificity
Hizentra	Normal human IgG	-
Cytogam	Human IgG against HCMV	-
6B4	Monoclonal antibody	gB
2B11	Monoclonal antibody	gB
11B12	Monoclonal antibody	gH
8I21	Monoclonal antibody	gH/gL/UL128/130

2.2.4 Clinical study populations and sample collection

To test if wild-type HCMV strains can be neutralized by immunoglobulin or monoclonal antibodies, samples were collected from HCMV infected individuals to obtain wild-type HCMV virions. The Health Research Ethics Board of the University of Alberta approved the use of samples for this study.

Participants included in this study were from four subpopulations: primary infected pregnant women, congenitally infected infants, transplant patients, and breastfeeding mother-infant pairs. The congenital infection group (primarily infected mothers and congenitally infected infants) were recruited based on serostatus of mothers and HCMV DNA levels in infants' urine (>10^6 IU/ml); infants in the breastfeeding group were between 1 to 8 months old and had received breast milk from HCMV seropositive mothers; transplant patients (HCMV D+/R-) after antiviral prophylaxis were recruited for the transplant group.

Urine and saliva of all infected individuals with the addition of breast milk from breastfeeding mothers were collected. Urine and saliva of adult participants were collected in 60ml sterile collection containers (StarPlex Scientific, US) and 15ml sterile centrifuge conical tubes (Corning, US) respectively. Additional breast milk was collected by 60ml sterile collection containers. Sterile cotton balls were put on breastfed infants' diapers in order to soak up urine and stored in 60ml sterile collection containers during transportation. Infants' saliva was collected by SalivaBio infant's swabs (Stratech Scientific, Australia) using the manufacturer's instructions. After arriving at the research laboratory, the urine and saliva were squeezed from cotton balls and swabs to multiple self-standing 5ml screw cap centrifuge tubes (Corning, US) using 10ml syringes (Becton Dickinson, US).

All samples were screened by the laboratory-developed qPCR (described in section 2.3.4), and every sample with a cycle threshold (Ct) value ≤33 determined by qPCR

would be further tested by the immunostaining-based neutralization assay (described in section 2.3.3) and the RT-qPCR-based neutralization assay (described in section 2.3.6).

Sample collection for transplant patients started after antiviral prophylaxis medication for HCMV and was conducted every week until the 12th week. For mother-infant pairs, samples of mother and baby in the same pair group were collected at the same time every month until the baby was 8 months old unless otherwise indicated. All samples were kept in thermal bags with ice packs during transportation. Selected samples with a good Ct value were analyzed by neutralization test right after qPCR screening. The rest of the samples were stored at -70℃ until further use.

2.3 Methods

2.3.1 Cell culture and preparation of viral stock

MRC-5, ARPE-19 and HMEC-1 were recovered from liquid nitrogen and seeded in 75 cm^2 flasks (Tissue Culture Treated T75, BD Falcon, US) with growth medium. After a one-week incubation in a 37℃ humidified 2.5% CO_2 incubator, cells in each flask were trypsinized to obtain a single-cell suspension and reseeded in culture plates depending on the need for each experiment. Seeding density of each cell type in different cultureware is shown in Table 2.2. After seeding, cells were incubated with growth medium in a 37℃ humidified 2.5% CO_2 incubator until 85% of cell confluency was reached for use.

HCMV can grow rapidly and quickly mutate in fibroblasts. To prevent further mutations in VR1814, ARPE-19 was selected as the cultivation cell line for viral culture. An 80% confluency of ARPE-19 monolayers was inoculated by VR1814 in a 75 cm^2 flask and then inoculated with maintenance medium in a 37℃ humidified 2.5% CO_2 incubator until 100% cytopathogenic effect (CPE) was observed. The flask was frozen and thawed for three cycles to lyse cells and therefore released viral particles. VR1814 virions were gently suspended in the medium, pipetted as 1 ml aliquots into screw cap microfuge tubes (Fisher Scientific, US) and stored at -70℃ for viral stock.

Table 2.2 Seeding density of each cell type in different cultureware

	Seeding density (cells/well or cells/flask)		
	75 cm^2 flasks	96-well plates	24-well plates
MRC-5	2×10^6	2.5×10^4	1.25×10^5
ARPE-19	2×10^6	2×10^4	1×10^5
HMEC-1	2×10^6	1.5×10^4	7.5×10^4

2.3.2 Viral stock quantification by plaque reduction assay

A plaque reduction assay previously described by Wentworth *et al*. was used to quantify infectious virions of viral stock in MRC-5 (129). Briefly, the viral stock was thawed, gently resuspended, and serially diluted 10-fold with maintenance medium. MRC-5 cells in a 24-well plate were inoculated by 1 ml of each dilution per well in triplicate. Three wells of cells were covered by maintenance medium only as negative controls. After a 1-hour incubation at 37°C, maintenance medium of each well was replaced with 2 ml of overlay containing maintenance medium with 0.15% agarose (Invitrogen™ UltraPure™ Agarose, Fisher Scientific, US). The plate was incubated at 37°C under 2.5% CO_2 for 7 days. A second overlay with the same ingredients was placed above the first overlay. After another 7-day incubation, 2 ml of 10% formalin was added to each well to fix monolayers for 30 min. Overlays of each well were discarded. Formalin was aspirated. Monolayers were washed by phosphate-buffered saline (PBS) three times and stained by immunoperoxidase staining assay (details described in section 2.3.3).

Negative controls were carefully checked under a light microscope to ensure that there were no stained spots. The number of stained plaques was counted and averaged for each dilution. Wells with more than 50 stained plaques which led to uncountable overlapped plaques were not counted. The titer of virus stock was calculated by the equation as follows: $Viral\ titer = \frac{Number\ of\ stained\ plaques}{d \times v}$, where d represents the dilution and v represents the volume of diluted viruses added to the well. After the plaque reduction assay, the titer of viral stock was 1.23×10^6 plaque-forming unit (PFU)/ml in MRC-5 cells.

2.3.3 Immunoperoxidase staining assay[1]

The immunoperoxidase staining assay was originally described by Anna Maria Abai *et al*. with a few modifications (82). Briefly, after HCMV inoculation, monolayers of each well were fixed with 150 µl of absolute ethanol for 30 min and then exposed to 150 µl of PBS for rehydration for 10 min, followed by a 30 min incubation with 150 µl of 5% normal goat serum (Normal goat serum ab7481, Abcam Inc, Canada) diluted

[1] Immunostaining for neutrazation tests conducted on wild-type HCMV in clinical specimens was performed by Dr. Maria Eloisa Hasing Rodriguez (Postdoctoral Fellow, Department of Laboratory Medicine and Pathology, University of Alberta)

with PBS to prevent non-specific background staining. 100 µl of 0.1 µg/ml of primary antibody anti-CMV IE1 monoclonal IgG (clone 8B1.2, Millipore Corporation, Canada) and 100 µl of a secondary antibody 5/10000 dilution of biotin-conjugated goat anti-mouse IgG (Invitrogen™, Fisher Scientific, US) with PBS supplemented with 5% casein blocker (Blocker™ casein, ThermoFisher Scientific, US) were successively added to each well and incubated for 1 hour and 30 min respectively. After antibody binding steps, 100 µl of a 2 µg/ml of HRP-Conjugated Streptavidin (Invitrogen™, Fisher Scientific, US) diluted with PBS supplemented with 5% casein blocker was added to each well for 30 min followed by 100 µl of TrueBlue (TrueBlue™ Peroxidase Substrate; Seracare Life Sciences Inc, US) for 15 min. After staining, monolayers were rinsed with distilled water followed by absolute ethanol and then dried inside the hood for 10 min. The entire staining procedure was conducted at room temperature and monolayers were washed three times with PBS before each step.

Stained spots or plaques were scanned with an ImmunoSpot Analyzer from C.T.L (Cellular Technology Limited, US) and counted manually. Negative controls were carefully checked under a light microscope.

2.3.4 Quantitative PCR[2]

The laboratory-developed qPCR described by Pang *et al*. was used to screen clinical samples and generate lab strain viral proliferation kinetics in this study (130).

Total nucleic acid was extracted from 200 µl of samples or harvested cell-medium mixture in each well by QIAamp DNA Mini Kit (Qiagen, US) and eluted with 100 µl of elution buffer using the manufacturer's instructions. Distilled water and VR1814 were included in each extraction as negative and positive extraction controls. Extracted nucleic acid was stored at -70°C until use or analyzed immediately.

20 µl of PCR mixture consisting of 10 µl of nucleic acid solution, 4 mM of $MgCl_2$, 0.5 µM concentrations of each primer, 0.2 µM concentrations of each probe, and 2 µl of the reagent from a LC-FastStart DNA Master hybridization probe kit (Roche Diagnostics, US) was added to the capillaries (Roche Diagnostics, US). The PCR experiment was performed on a ROCHE LightCycler® 2.0 Instrument (Roche Diagnostics, US) with LightCycler® Software 4.1. The thermal cycling protocol was a

[2] The author would like to acknowledge laboratory technician Min Cao, Postdoctoral Fellow Dr. Maria Eloisa Hasing Rodriguez, and Postdoctoral Fellow Dr. Sudha Bhavanam for the help with qPCR analysis.

10 min initial interval at 95°C followed by an amplification procedure consisting of 45 cycles of 15 s denaturation at 95°C, 10 s annealing at 55°C, and a 10 s extension at 72°C. DNA extracted from VR1814 and distilled water were included in each qPCR run as positive and negative controls to ensure that the results of qPCR were reliable. A standard was included in each qPCR run for the quantification measurement.

In order to determine the specificity of the RT-qPCR assay, isolated RNA-free DNA was obtained by adding 4 µl of RNase A (100 mg/ml, Thermo Fisher Scientific, US) prior to the addition of Buffer AL using the manufacturer's instructions.

2.3.5 RNA sample preparation with or without RNA extraction

2.3.5.1 Nucleic acid extraction by commercial kits

A 100 µl volume of BL+TG buffer from MagaZorb® Total RNA Mini-Prep Kits (Promega, US) was added to each well of 96-well plates and incubated for 2 min at room temperature. The mixture in each well was transferred to Eppendorf microcentrifuge tubes and extracted by MagaZorb® Total RNA Mini-Prep Kits using the manufacturer's instructions. A 50 µl volume of extracted nucleic acid was eluted with nuclease-free water and stored at -70℃ until RT-qPCR analysis. Distilled water and VR1814 were extracted as negative and positive controls respectively.

2.3.5.2 RT-qPCR-ready cell lysates without RNA extraction

An RT-qPCR-ready cell lysate generation method originally designed by Kenneth Shatzkes *et al*. was optimized and evaluated in this study (112). After optimization, 100 µl of CL buffer containing 10mM Tris-HCl, pH 7.4, 0.25% Igepal CA-630 and 150 mM NaCl was added to each well of 96-well plates and incubated for 10 min at room temperature. 100 µl of the mixture in each well was transferred to Eppendorf microcentrifuge tubes and spun on a medium setting (setting 6 out of 8) of a vortex mixer (Fisher Scientific, US) for 30 seconds followed by a 15000 relative centrifugal force (rcf) centrifugation at 20℃ for 2 min. After centrifugation, supernatants were carefully collected without disturbing the pellet and transferred to new 1.5 ml screw-cap tubes. The collected cell lysates were either tested in fresh or stored at -70℃ until RT-qPCR assessment.

2.3.5.3 Optimization of the RT-qPCR-ready cell lysates

Inhibition was observed when the original RT-qPCR-ready cell lysate generation method was applied to the RT-qPCR assay for HCMV neutralization, leading to questions of how to eliminate inhibitors in cell lysates and whether the lysing capacity of the in-house cell lysis (CL) buffer met the need of this study.

First, the source of inhibition was tracked. 1 ml of trypsin EDTA solution (1x Liquid 0.25% Trypsin/1mM EDTA; Gibco®, Thermo Fisher Scientific, US) was added to a 75 cm^2 tissue culture flask of cell stocks to disaggregate monolayers. Medium with cells was gently pipetted in order to obtain single-cell suspensions. The number of cells in the suspension was counted, and a series of cell dilutions at concentrations of 1000 cells/µl, 800 cells/µl, 600 cells/µl, 400 cells/µl and 200 cells/µl were generated with PBS. 50 µl of each dilution was transferred to an Eppendorf tube. Cell suspensions were centrifuged at 2000 rcf for 4 min at 20℃ and supernatants were aspirated without disturbing cell pellets. Pellets were resuspended in 95 µl of CL buffer containing 10mM Tris-HCl, pH 7.4, 0.25% Igepal CA-630 and 150 mM NaCl and incubated at room temperature for 10 min. CL buffer was prepared as the CL buffer group. Nuclease-free water was prepared as the control group and used as the baseline of RNA levels spiked in each group. 5 µl of extracted VR1814 RNA was spiked to each tube and carefully mixed. 2 µl of each mixture was analyzed by RT-qPCR. All tests were conducted in triplicate by using MRC-5, ARPE-19 and HMEC-1 cells. The mean of IU/reaction for each condition was calculated and a bar chart was generated to determine whether the RT-qPCR inhibitors came from cell lysates or CL buffer itself.

After identifying cell lysates as the source of inhibition, an additional centrifugation step at different centrifugal force settings (5000 rcf, 10000 rcf and 15000 rcf at 20℃ for 2 min) was added after cell lysis in order to reduce amplification inhibition. All tests were conducted in triplicate by using MRC-5, ARPE-19 and HMEC-1 cells. The mean of IU/reaction for each condition was calculated and a bar chart was generated to determine if centrifugation helped reduce RT-qPCR inhibition.

In order to improve the cell lysing capacity of CL buffer, an additional vortex step was added after CL buffer incubation. 6000 PFU/well of VR1814 was used to inoculate cells in 96-well plates with maintenance medium. After 20-hour incubation in a 37℃ humidified 2.5% CO_2 incubator, monolayers were exposed to 100 µl/well of CL buffer for 10 min at room temperature. Cell lysates of each well were transferred to Eppendorf

tubes, followed by vortex on different settings, including low setting (3 out of 8) for 30 seconds, low setting (3 out of 8) for 1 min, medium setting (6 out of 8) for 30 seconds and medium setting (6 out of 8) for 1 min. Each condition was measured in triplicate and three tubes of cell lysates without vortex were defined as a non-vortex group. The test was conducted in MRC-5, ARPE-19 and HMEC-1. All groups of cell lysates were centrifuged at 15000 rcf for 2 min at 20℃ before RT-qPCR analysis. The mean of IU/reaction for each vortex setting was calculated and a bar chart was generated to compare the impact of different vortex settings to RT-qPCR assessment.

Additionally, the lysing capacity of the optimized cell-lysate generation method was determined. Cell pellets (250,000, 50,000, 10,000, 2,000, 400 cells/pellet) were obtained in Eppendorf tubes the same way as previously described and exposed to 100 µl of CL buffer at room temperature for 10 min, followed by vortex on medium setting (6 out of 8) for 30 seconds and centrifugation at 2000 rcf for 4 min at 20℃. After centrifugation, cell pellets were resuspended with 10 µl of PBS. The number of remaining entire cells was counted manually using a hemocytometer (0.1mm deep, Hausser Scientific, US) under a light microscope.

Finally, the optimized cell-lysate generation method was compared with a commercial RNA extraction kit from MagaZorb to determine whether the optimized cell lysates could replace RNA extraction for RT-qPCR assessment.

A 10-fold serial dilution of VR1814 from 10^4 to 1 PFU/well in maintenance medium was used to inoculate cells in two 96-well plates. Maintenance medium was used as negative controls. After a 20-hour incubation at 37℃ under 2.5% CO_2, two plates were processed by MagaZorb® Total RNA Mini-Prep Kits and 100 µl of modified CL buffer respectively, followed by RT-qPCR analysis in same RT-qPCR run. The test was measured in triplicate and conducted in MRC-5, ARPE-19, and HMEC-1.

Data from the RNA extraction group was plotted on the y-axis against data from the cell lysate group on the x-axis, and a line of equality was drawn to test the degree of agreement between two methods. A correlation coefficient (*r*) was calculated using the equation as follow: $r_{xy} = \frac{\sum_{i=1}^{n} (x_i-\bar{x})(y_i-\bar{y})}{\sqrt{\sum_{i=1}^{n} (x_i-\bar{x})^2}\sqrt{\sum_{i=1}^{n} (y_i-\bar{y})^2}}$, where n is the sample size, $\bar{x}, \bar{y}$ are sample means, and x_i, y_i are individual sample points. A paired *t*-test was used to determine if HCMV RNA yielded by two methods were statistically different.

2.3.6 One-step RT-qPCR assay

The SYBR Green-based RT-qPCR assay for quantitation of HCMV replication was previously described by Xiao Wang *et al*(97). Briefly, primers AGATGTCCTGGCAGAACTCGTC (forward) and TTCTATGCCGCACCATGTCCAC (reverse) purchased from IDT (Integrated DNA Technologies, US) were used to target a 62 bp mRNA segment encoded by IE gene IE1 (UL123). The UL123 primers were selected to span an exon-exon junction from exon 3 to exon 4 in the UL123 gene in order to avoid the effect of non-specific products (e.g. genomic DNA from cells and viruses) and simplify the experimental procedure by skipping DNase treatment.

RT-qPCR was carried out by iScript One-Step RT-PCR kits (Bio-Rad) according to the manual. The final RT-qPCR mixture contained 10 µl of iTaq universal SYBR® Green reaction mix (2x), 0.25 µl of iScript reverse transcriptase, 300 nM concentrations of each primer, 2 µl of cell lysate, and nuclease-free H_2O to 20 µl. The thermal cycles were performed on an Applied Biosystems 7500 Fast Real-Time PCR System (ABI, US) under standard mode as follows: 10 min at 50℃ for the reverse transcription reaction; 1 min at 95℃ as the initial stage of PCR for polymerase activation, followed by 40 cycles of amplification consisting of 15 sec denaturation at 95℃ and 60 sec extension at 60℃, and a default setting of melt-curve analysis at the end. Plate reading was conducted during the extension period. Since SYBR® Green dye used in this RT-qPCR assay could nonspecifically bind to any double-strand DNA, melting curve analysis was performed and melting temperature (Tm) within the range of 76.8 to 78.8℃ was considered as Tm for true positive results.

RNA extracted from VR1814 and nuclease-free water were used as positive and negative controls in each RT-qPCR run. A standard was included in each RT-qPCR run for quantification assessment.

2.3.6.1 Standard control development and standard curve establishment

The standard control used for RT-qPCR was produced from VR1814 viral stock which has been quantified as PFU per ml and extracted by MagaZorb® Total RNA Mini-Prep Kits. A 10-fold serial dilution of extracted RNA from 0.5 PFU/µl (1 PFU/reaction) to 5000 PFU/µl (1×10^4 PFU/reaction) was analyzed by the one-step RT-qPCR. The rest of the undiluted RNA was aliquoted and stored at -70℃ for setting up

external standards for each RT-qPCR assessment.

The standard curves were generated by plotting the average Ct values obtained in two different days on the y-axis against the log (base 10) scale of HCMV infectious titers in each dilution on the x-axis. A linear regression analysis was performed to calculate the linear equation Y = a + bX, where b was the slope used to determine the efficiency of the assay and a was the y-intercept. R^2 was also calculated to evaluate if the results of RT-qPCR could be directly proportional to infectious particles (IU/ml) in samples. The results of RT-qPCR were converted from Ct value into HCMV IU/reaction based on the equation established by the standard curve. Any results with a Ct value above 34 or a Tm outside the range (76.8 to 78.8°C) were defined as negative.

2.3.7 Evaluation of RT-qPCR for HCMV quantification in viral culture

2.3.7.1 Sensitivity

Five concentrations (0.5, 0.4, 0.3, 0.2, 0.1 PFU/μl) of extracted RNA near the expected limit of detection (LOD) were produced from quantified VR1814 viral stock and analyzed by the RT-qPCR eight times on different days. Probit analysis was applied to analyze the relationship between the concentration of interest and two possible outcomes (positive/negative). For each concentration, the detection probability was calculated by the equation: $D_i = \frac{n_{pos}}{n_{neg}}$, where n_{pos} represents the number of positive RT-qPCR results and n_{neg} represents the number of negative RT-qPCR results. Detection probabilities were plotted against concentrations and a probit analysis (STATA15.1, StataCorp LLC, US) was applied to the graph. The concentration equivalent to 95% detection probability was reported as the LOD of the RT-qPCR.

2.3.7.2 Specificity

Five RNA-free DNA samples extracted from VR1814 were tested by the RT-qPCR to determine if the HCMV DNA can be detected by the RT-qPCR. Extracted RNA from VR1814 and distilled water were used as positive and negative controls.

2.3.7.3 Precision

Nine HCMV positive samples with expected RNA levels (three high positives, three medium positives and three low positives) were selected to evaluate the precision of the RT-qPCR. The expected RNA levels were estimated based on the results of qPCR.

Extracted RNA of each sample was divided into three aliquots and stored at -70℃ until use. Each aliquot was thawed at room temperature for 15 min and tested in triplicate by the RT-qPCR under the same condition on three different days. Mean, standard deviation (SD) and coefficient of variation (CV) were calculated to evaluate the intra- and inter-assay precision.

2.3.7.4 The kinetics of VR1814 proliferation in viral culture

1000 PFU/well of VR1814 diluted with maintenance medium was used to inoculate MRC-5, ARPE-19, and HMEC-1 in two 96-well plates and incubated at 37℃ under 2.5% CO_2. Medium and cells of each well were collected at 0, 1, 3, 5, 8, 10, 13, 16, 18, 22, and 54 h.p.i. and analyzed by qPCR and RT-qPCR parallel. Maintenance medium for each cell type was used as negative controls. All tests were conducted in triplicate.

A log (base 10) scale of average HCMV copy numbers/well determined by qPCR was plotted on the y-axis against the hours of post-infection on the x-axis to generate HCMV DNA accumulation curves, while a log (base 10) scale of average HCMV IU/well determined by RT-qPCR was plotted on the y-axis against the hours of post-infection on the x-axis to generate HCMV gene expression curves.

2.3.7.5 Timepoint optimization for RT-qPCR on measuring wild-type HCMV infectivity

To determine if the timepoint 20 h.p.i. for RT-qPCR on VR1814 was also appropriate for wild-type HCMV strains in primary clinical samples, a total of six HCMV DNA-positive clinical samples including one saliva, two urine and three breast milk were selected in this validation. 200 µl of each sample was used to inoculate MRC-5, ARPE-19, and HMEC-1 in four replicates in 96-well plates. Two replicates of each sample were harvested at 20 h.p.i. and the other two replicates at 48 h.p.i.. Growth medium for each cell type was used as negative controls and all samples were analyzed by RT-qPCR in the same run.

A paired *t*-test was applied to analyze if there was a significant difference between results obtained at the two time points by the equation: $t = \frac{\bar{x}_D}{\frac{s_D}{\sqrt{n}}}$, where $\bar{x}_D$ represents the mean of differences between each pair, s_D represents the standard deviation of differences between each pair, and n represents the number of pairs. The confidence level is 95% for the paired *t*-test so that the definition of statistical significance was *P*

< 0.05.

2.3.8 Neutralization assay for HCMV

2.3.8.1 Clinical sample preparation

In order to avoid potential cell culture contamination caused by microorganisms from clinical samples, antibiotic and antifungal medicines were added to clinical samples and growth medium prior to being used as inoculations in neutralization tests. Some contaminants (e.g. fat, cell debris) in clinical samples were also removed before analysis.

0.5% of Fungin (10 mg/ml, InvivoGen, US) and 5% of Penicillin-Streptomycin (10,000 U/ml, ThermoFisher Scientific, US) were added to each clinical sample. All samples were diluted with 1.5 times of growth medium supplemented with 1% of penicillin-streptomycin and 0.1% of Fungin unless otherwise indicated. The pH of each diluted sample was then adjusted to 7.4 with hydrochloric acid (Fisher Scientific, US) or sodium hydroxide (Fisher Scientific, US).

Prior to neutralization tests, breast milk was centrifuged at 14000 rpm for 5 min at 20℃ so that fat could be separated on the top from other compounds in breast milk and removed using a 100 µl pipette (Pipet-Lite™ XLS, Rainin, US). Saliva was also centrifuged at 10000rpm for 2 min at 20℃ to remove cell debris. The supernatants were carefully collected without disturbing pellets and transferred to new 1.5 ml screw-cap tubes for further analysis.

2.3.8.2 Neutralization test for HCMV

Clinical samples were screened and selected as described in Section 2.2.4. Monolayers of three cell lines were prepared as described in Section 2.3.1. 850 µl of 2-fold serial dilutions of HIG Hizentra (50 mg/ml to 0.38 µg/ml) made by growth medium were mixed with an equal volume of samples (1000 PFU/well of VR1814 or 850 µl of clinical samples) and incubated for 1 hour at 37℃. 200 µl of the antibody-virus mixture was added to each well of 96-well plates followed by centrifugation at 300 rcf for 30 min. the plates were incubated at 37℃ under 2.5% CO_2 for 20 hours for lab-strain neutralization test or 48 hours for clinical-strain neutralization test unless otherwise indicated. 850 µl of VR1814 and growth medium were used as positive and negative controls. All tests were measured in triplicate.

After incubation, plates were analyzed by immunostaining or RT-qPCR as described in section 2.3.3 and 2.3.6 respectively. The percentage of neutralization was calculated by the equation: $\%Neutralization = (1 - \frac{Mean\ Infected\ Cells_{Test}}{Mean\ Infected\ Cells_{baseline}}) \times 100$, where Mean Infected $Cells_{Test}$ was the average number of infectious units in each antibody dilution, and Mean Infected $Cells_{Test}$ was the average number of infectious units in positive controls where antibodies were absent. The percentage of neutralization was plotted on the y-axis against a log (base 10) scale of antibody dilutions on the x-axis. Neutralization curves were generated by GraphPad Prism 8 (GraphPad Software, San Diego, US) in the Sigmoidal 4-PL model with constraining the top value as 100 and the bottom value as 0. The fifty percent antibody neutralizing titer (IC50 in Prism 8) was the midpoint of the neutralization curve and calculated automatically by the software.

2.3.9 Comparison of the RT-qPCR assay with the immunostaining assay in HCMV infectivity assessment

First, the correlation between RT-qPCR and immunostaining on detecting HCMV infectious titers was determined based on Pearson's correlation coefficient. A series of diluted VR1814 (25, 50, 100, 200, 500, 1000, 1500, 2000 PFU/well) in maintenance medium was used to inoculate MRC-5s in two 96-well plates. After 24-hour incubation in a 37℃ humidified 2.5% CO_2 incubator, one plate was analyzed by the RT-qPCR and another by the immunostaining. Each dilution was measured in six replicates and MRC-5 maintenance medium was used as negative controls. Data from the immunostaining was plotted on the y-axis against data from the RT-qPCR on the x-axis, and a regression line was drawn to test the degree of agreement between two methods. A correlation coefficient (r) was calculated as previously described.

Second, the sensitivity and specificity of RT-qPCR and immunostaining on detecting HCMV infectious titers were compared. Three concentrations (1, 10, 20 PFU/well) of diluted VR1814 in maintenance medium were used to inoculate cells in two 96-well plates. After 24-hour incubation, one plate was analyzed by the RT-qPCR and another by immunostaining. Maintenance medium for each cell type was used as negative controls. Each concentration including the negative control was measured in six replicates in MRC-5, ARPE-19, and HMEC-1. The limit of detection was analyzed by Probit analysis as described in section 2.3.7.1.

Third, the RT-qPCR-based and immunostaining-based neutralization assays were applied to HCMV lab strain VR1814 against human normal immunoglobulin Hizentra in order to determine the agreement of two assays on neutralization. A 2-fold serial dilution of Hizentra from 50 mg/ml to 0.38 µg/ml was prepared in maintenance medium. 850 µl of each immunoglobulin dilution was mixed with an equal volume of VR1814. VR1814 in absence of Hizentra was used as positive controls and maintenance medium as negative controls. The neutralization test procedure, generation of neutralization curves, and calculation of IC50 values were performed as described in section 2.3.8.2.

Finally, the agreement of two assays was determined on assessing the infectivity of wild-type HCMV virions. Urine, saliva and breast milk clinical samples were screened by qPCR and prepared for cell culture as described in section 2.2.4 and 2.3.8.1. 200 µl of selected clinical samples were used to inoculate MRC-5, ARPE-19, and HMEC-1 in 96-well plates. Plates were incubated in a 37°C humidified 2.5% CO_2 incubator for 48 hours and analyzed by immunostaining and RT-qPCR respectively. VR1814 and maintenance medium of each cell type were used as positive and negative controls. Fisher's exact test was applied to analyze if the performances of two assays were the same using the equation: $p = \frac{(a+b)!(c+d)!(a+c)!(b+d)!}{a!b!c!d!n!}$, where a represents the number of samples detected as positive by both assays, b represents the number of samples detected as negative by RT-qPCR but positive by immunostaining, c represents the number of samples detected as positive by RT-qPCR but negative by immunostaining, and d represents the number of samples detected as negative by both assays. The confidence level is 95% for Fisher's exact test so that the definition of statistical significance was $P < 0.05$.

2.3.10 Antibody neutralization of wild-type HCMV from clinical specimens

A urine sample from an 8-month-old baby with a qPCR Ct value at 29.1 and a saliva sample from a 4-month-old baby with a qPCR Ct value at 14.81 were selected to test the neutralizing activity of a panel of antibodies in MRC-5. The antibody panel included HIG Cytogam and monoclonal antibodies 6B4, 2B11, 11B12, 8l21. Two concentrations of antibodies were used. The low concentrations were selected based on the IC90 values for VR1814 in fibroblasts previously described by Annalisa Macagno *et al*.(131): Cytogam (640 µg/ml for urine or 1280 µg/ml for saliva), 10 µg/ml for 6B4, 7.5 µg/ml for 2B11, 35 µg/ml for 11B12, and 25 µg/ml for 8l21. The high concentrations

of immunoglobulin and antibodies were chosen based on the concentrations used by Cui *et al.*(43): Cytogam (1280 µg/ml for urine or 2100 µg/ml for saliva) and 50 µg/ml for 6B4, 2B11, 11B12, and 8l21. 850 µl of each concentration of antibodies was mixed with an equal volume of clinical samples. Clinical samples in absence of antibodies were used as positive controls and growth medium as negative controls. The neutralization test was performed as described in section 2.3.8.2, and a neutralization test on VR1814 under the same conditions was also conducted in order to compare if lab strains had the same neutralization resistance as wild-type strains. The whole experiment was conducted in MRC-5, ARPE-19 and HMEC-1, and analyzed by both immunostaining-based and RT-qPCR-based neutralization assays at 48 h.p.i.. A vertical bar chart depicting the percentages of neutralization was generated in order to compare the neutralization differences of each antibody against wild-type HCMV in primary clinical samples and VR1814.

HCMV DNA-positive clinical samples with either a medium HCMV DNA level or limited sample volume were neutralized only with Cytogam at the concentration of 1280 and 640 µg/ml. The neutralization test was conducted as described in section 2.3.9. A table depicting the percentages of neutralization was generated to show neutralizing activity of HIG against wild-type HCMV in primary clinical samples.

2.3.11 Statistical analysis

In this study, descriptive statistics (e.g. mean, SD, CV), bar charts and line charts were obtained from Microsoft Excel (Microsoft Office 365 Proplus). Statistical analyses were described in each section and performed using GraphPad Prism 8 (GraphPad Software, San Diego, US). All *P*-values reported were two-tailed with a 95% confidence interval unless otherwise indicated.

Chapter 3 Results

3.1 Evaluation of the one-step RT-qPCR assay

3.1.1 Standard curve establishment

The one-step RT-qPCR assay was designed to be capable of detection of HCMV replication and be able to estimate the titer of infectious HCMV virions in the sample of interest.

The RT-qPCR was shown to have a dynamic range from 1 to 1×10^4 PFU/reaction (Figure 3.1). A graph of the amplification plot against its molecular standard generated by the Applied Biosystems 7500 Fast Real-Time PCR System is shown in Figure 3.2.

The R^2 value of 0.9994 provides good confidence in Ct value and infectious units, indicating that the Ct value can be used to accurately predict the value of infectious units. Therefore, the Ct value of RT-qPCR was converted into infectious units in this thesis using the equation by linear regression analysis as follow: $Y = \frac{33.977-X}{3.3864}$, where Y is the $\log_{10}$IU/reaction and X is the Ct value provided by the RT-qPCR assay. Based on the equation, a Ct value of 34 was defined as the cut-off point for positive RT-qPCR results, as Ct values above 34 indicate that the infectious virions detected by RT-qPCR were less than one infectious particle per reaction.

To identify true positive RT-qPCR results, Tm was another important parameter for SYBR green-based RT-qPCR assays. The average Tm of standard controls (77.8℃) was defined as the Tm of amplicon for the RT-qPCR assay, and Tm ± 1℃ (76.8℃≤Tm≤78.8℃) was recognized as Tm range to distinguish specific and nonspecific PCR products (Figures 3.3).

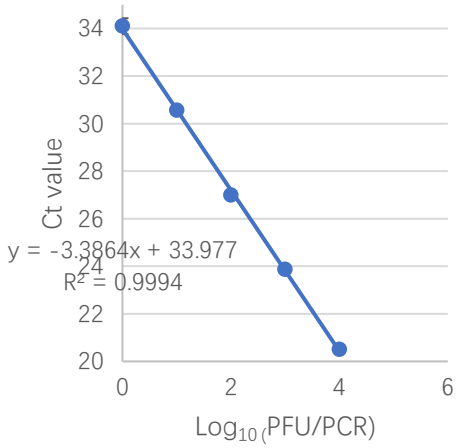


Figure 3.1 A standard curve of the one-step RT-qPCR assay
The standard RNA from the HCMV immediate-early gene UL123 was extracted from 10-fold serial dilutions of HCMV ranging from 1 to 10^4 PFU/reaction and amplified in the one-step RT-qPCR assay. The quantity of infectious particles was plotted against average cycle numbers.

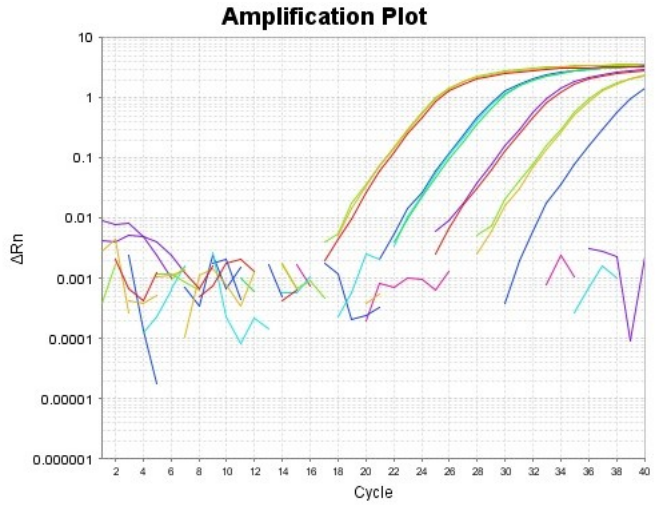


Figure 3.2 Amplification plot of 10-fold serial dilutions by the one-step RT-qPCR assay
Amplification plot of 10-fold serial dilutions of standard RNA ranging from 1 to 10^4 PFU/reaction generated by the one-step RT-qPCR assay.

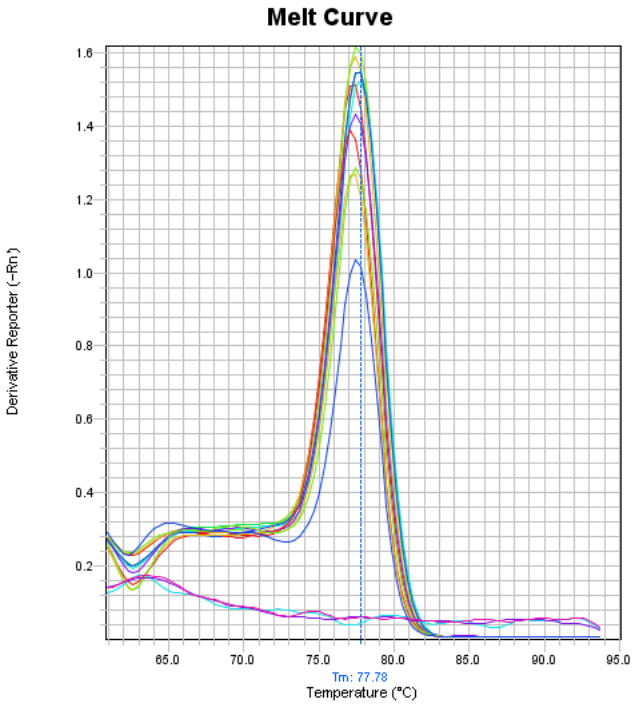


Figure 3.3 Melt curves of standard controls by the one-step RT-qPCR assay
Melt curves of standard RNA ranging from 1 to 10^4 PFU/reaction determined by the one-step RT-qPCR assay.

3.1.2 Sensitivity

The LOD of the RT-qPCR assay was determined by a series of diluted standards. Dilutions at 0.6, 0.8, 1 PFU/reaction were detected in all eight runs (100%), but 0.4 PFU/reaction was only detected in six out of eight RT-qPCR reactions, giving a 75%

positive rate. The LOD of the assay was defined as 0.6 PFU/reaction (Table 3.1).

Table 3.1 Limit of detection of the one-step RT-qPCR assay

PFU/PCR	No. of Pos specimens/amount tested	%Positive
1	8/8	100.0
0.8	8/8	100.0
0.6	8/8	100.0
0.4	6/8	75.0
0.2	1/8	12.5

3.1.3 Specificity

No RT-qPCR signal was generated in five RNA-free DNA samples, indicating that the RT-qPCR assay for HCMV RNA detection had no cross-reactivity with viral DNA.

3.1.4 Precision

Both intra- and inter-run precision of the one-step RT-qPCR assay were evaluated based on the mean, standard deviation and coefficient of variation of nine HCMV-positive samples.

For intra-run precision, the mean of $\log_{10}$IU/reaction was obtained from 3 replicates of each sample on the same day (Table 3.2). The percentages of CV range from 0.38% to 9.41%. The highest CV (9.41%) was from the sample with the lowest viral load.

For inter-run precision, the mean of $\log_{10}$IU/reaction was obtained from 9 replicates of each sample detected on three different days (Table 3.3). The percentages of CV range from 0.94% to 15.08%. Variation in the inter-precision test was slightly higher than the intra-precision test. Similar to the intra-precision test, High CVs were also observed from the low viral load samples near the limit of detection of RT-qPCR.

Table 3.2 Intra-run precision of the one-step RT-qPCR assay

	No. of samples	Average $\log_{10}$ (PFU/reaction)	SD	%CV
High viral load 1	3	4.03	0.03	0.63
High viral load 2	3	2.42	0.01	0.38
High viral load 3	3	3.57	0.07	2.01
Medium viral load 1	3	2.57	0.03	1.33
Medium viral load 2	3	2.69	0.03	1.00
Medium viral load 3	3	1.47	0.13	1.24
Low viral load 1	3	0.66	0.02	3.29
Low viral load 2	3	1.07	0.04	3.76
Low viral load 3	3	0.64	0.08	9.41

Table 3.3 Inter-run precision of the one-step RT-qPCR assay

	No. of samples	Average $\log_{10}$ (PFU/reaction)	SD	%CV
High viral load 1	9	4.04	0.04	0.94
High viral load 2	9	2.48	0.11	4.25
High viral load 3	9	3.41	0.16	4.56
Medium viral load 1	9	2.46	0.19	7.87
Medium viral load 2	9	2.63	0.07	2.48
Medium viral load 3	9	1.54	0.08	5.32
Low viral load 1	9	0.72	0.10	13.31
Low viral load 2	9	1.01	0.08	7.40
Low viral load 3	9	0.71	0.11	15.08

3.1.5 The kinetics of VR1814 proliferation in viral culture

To select an endpoint that a sufficient amount of RNA had been yielded for RT-qPCR detection, kinetic curves of gene expression of VR1814 were generated in MRC-5, ARPE-19, and HMEC-1. VR1814 DNA growth curves were developed as well to determine if DNA can be an indicator of viral proliferation at an early stage.

Gene transcription of VR1814 increased rapidly in the first 5h.p.i. and then gradually entered a plateau in MRC-5, while RNA levels grew steadily until 16 h.p.i. in HMEC-1 and ARPE-19 (Figure 3.4A). The overall RNA accumulation of VR1814 reached a plateau at 20 h.p.i. in all three cell lines. In contrast, there was no significant change in viral DNA accumulation within 54 h.p.i. (Figure 3.4B), indicating that qPCR could not be used to measure HCMV infectivity at an early stage. Therefore, 20h.p.i. was defined as the time point to conduct RT-qPCR assessment for VR1814.

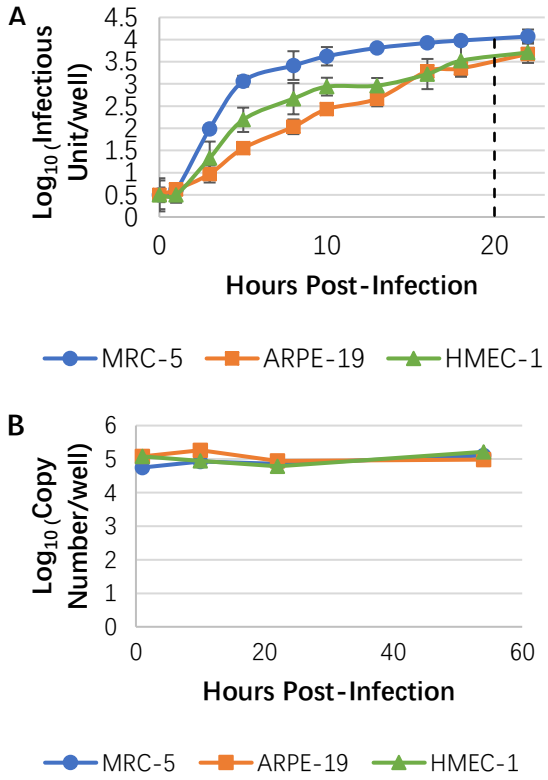


Figure 3.4 VR1814 proliferation in MRC-5, ARPE-19 and HMEC-1.
VR1814 was used to inoculate MRC-5, ARPE-19, and HMEC-1 cells in 96-well plates. (A) Medium and monolayers were collected at 0, 1, 3, 5, 8, 10, 13, 16, 18, and 22 h.p.i. and analyzed by RT-qPCR with primers targeting the HCMV IE1 gene transcript. (B) Medium and monolayers were collected at 1, 10, 22 and 54 h.p.i. and analyzed by qPCR with primers and probes targeting gB gene. Each point represents the mean with the standard deviation of the data set (n=4).

3.1.6 Timepoint optimization for RT-qPCR on measuring wild-type HCMV infectivity

Wild-type HCMV in six primary clinical samples were cultivated in MRC-5, ARPE-19 and HMEC-1 to investigate whether 20 h.p.i. could also be applied to RT-qPCR analysis on primary clinical samples. The Ct values of clinical samples at two different time points were very similar in MRC-5 (Table 3.4). In ARPE-19 and HMEC-1, false negative can be observed at both 20 h.p.i. in sample B056-3-URN and 48 h.p.i. in sample M053-1-BRM (Table 3.4). A relatively large variation between two timepoints can be observed in sample B056-4-SAL, where the Ct values in HMEC-1 were around 28 at 48 h.p.i. and 32 at 20 h.p.i., but the Ct values in ARPE-19 were the opposite. However, results obtained at 20 and 48 h.p.i. were not statistically different (P>0.05, paired t test), suggesting that 20 h.p.i. can be applied to RT-qPCR as the assessment time point for wild-type HCMV in primary clinical samples.

Table 3.4 Comparison of 20 and 48 h.p.i. for RT-qPCR assessment on wild-type HCMV in primary clinical samples

Clinical samples	Cell lines	48 hours post-infection (Ct value±SD)	20 hours post-infection (Ct value±SD)
M055-3-BRM	HMEC-1	Neg	Neg
	MRC-5	Neg	Neg
	ARPE-19	Neg	Neg
B056-3-SAL	HMEC-1	28.8±0.25	32.54±0.21
	MRC-5	21.43±0.12	21.76±0.06
	ARPE-19	32.34±0.66	28.33±0.12
B056-3-URN	HMEC-1	Neg	Neg
	MRC-5	30.64±0.75	30.89±0.47
	ARPE-19	33.1±0.06	Neg
M053-1-BRM	HMEC-1	Neg	33.4±0.47
	MRC-5	33.38[a]	32.66±0.49
	ARPE-19	32.61±0.02	32.74±0.24
M057-1-BRM	HMEC-1	Neg	Neg
	MRC-5	33.36±0.72	32.04±0.19
	ARPE-19	33.52[a]	32.79±0.03
B058-1-URN	HMEC-1	Neg	Neg
	MRC-5	Neg	Neg
	ARPE-19	32.81±1.23	32.36±0.25

[a] The sample had only one positive result in two replicates

3.2 Optimization of the RT-qPCR-ready cell lysates for RT-qPCR

3.2.1 Inhibition identification

RT-qPCR-ready cell lysates without RNA extraction can reduce running costs of RT-qPCR assessment, but false positive results could be observed (data not shown), indicating the presence of amplification inhibition in the RT-qPCR assay. Therefore, HCMV RNA was spiked to serially diluted cell lysates, CL buffer, and nuclease-free water to track the source of inhibitors.

VR1814 titers obtained from CL buffer and nuclease-free water were similar (1034 and 1000 IU/reaction respectively) (Figure 3.5), suggesting that CL buffer itself did not inhibit PCR amplification. In dilutions of cell lysates, detectable RNA level increased with the decrease in cell number, indicating that inhibitors were mainly from crude cell lysates.

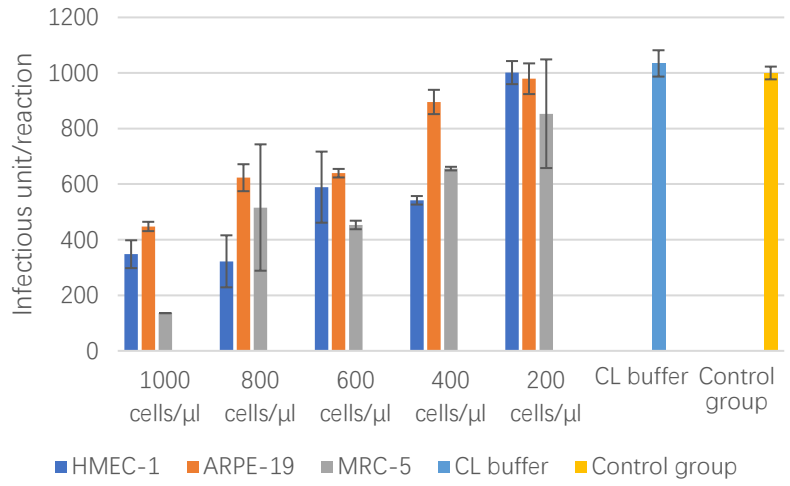


Figure 3.5 determination of the main source of inhibitors
Different concentrations of MRC-5, ARPE-19 and HMEC-1 cells were exposed to cell-lysis buffer (10mM Tris-HCl, pH 7.4, 0.25% Igepal CA-630 and 150 mM NaCl) for 10 minutes at room temperature and spiked with VR1814 RNA. 2 µl of each mixture was analyzed by RT-qPCR in comparison with data obtained from CL buffer and nuclease-free water group (n=3).

3.2.2 Inhibition reduction and HCMV RNA yield enhancement

To reduce amplification inhibition, an additional centrifugation step was added after cell lysis. RT-qPCR signals increased with the growing centrifugal force, especially in HMEC-1 (Figure 3.6). Detectable RNA levels in ARPE-19 and HMEC-1 (77 and 80 IU/reaction respectively) were close to the level in nuclease-free water (84 IU/reaction) at 15000 rcf, indicating that inhibition in crude cell lysates was reduced by centrifugation.

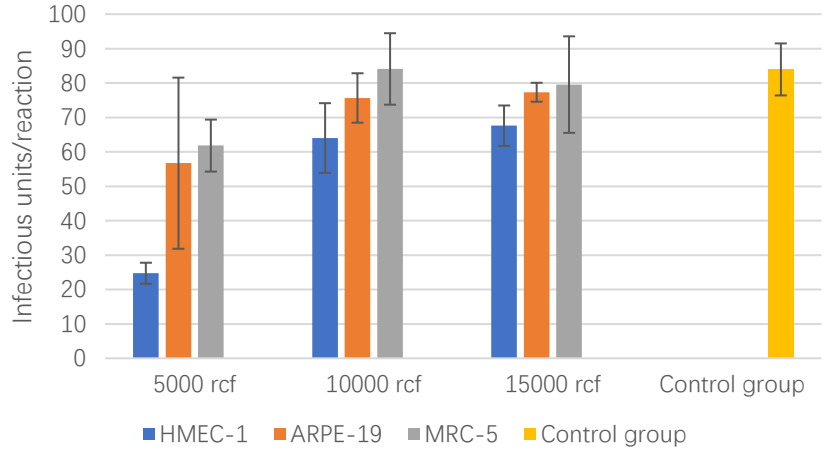


Figure 3.6 Effect of centrifugation on the reduction of inhibition
MRC-5, ARPE-19 and HMEC-1 cells were exposed to CL buffer at room temperature for 10 minutes and spiked with VR1814 RNA. 2 minutes of 5000, 10000, 15000 rcf centrifugation were applied to cell lysates respectively. 2 µl of supernatant was analyzed by RT-qPCR. The control group was nuclease-free water spiked with VR1814 RNA (n=3).

To further facilitate releasing viral RNA from cells and therefore increase RNA yield, a vortex step was added to help CL buffer lyse cells. RNA yields were almost doubled (from 3619.42 to 6116.26 IU/well) in MRC-5 after adding a medium vortex for 60 seconds (Figure3.7). A similar increase in RNA yields could also be observed in HMEC-1, but the RNA levels remained almost the same under different vortex settings in ARPE-19, indicating that the lysing capacity of CL buffer related to cell types and vortex enhanced RNA yields in HMEC-1 and ARPE-19. A slight decrease in RNA yields could also be observed under 60s medium vortex in HMEC-1 and ARPE-19, but the difference between the 30s and 60s vortex was not statistically significant (P>0.05).

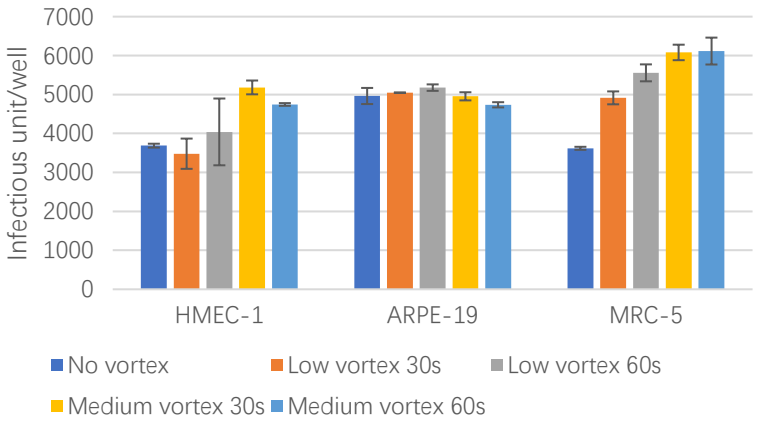


Figure 3.7 Effect of vortex on cell lysate preparation
VR1814 was used to inoculate MRC-5, ARPE-19 and HMEC-1. After 24 hour incubation, cell lysates were generated by adding 100 µl/well of CL buffer followed by vortex under different settings. 2 µl of cell lysate of each sample was analyzed by RT-qPCR (n=3).

The lysis ability of the modified CL buffer was further assessed. MRC-5, ARPE-19 and HMEC-1 cells below 500 cell/µl can be sufficiently lysed by the modified CL buffer (Table 3.5). Unlysed cells (192, 36 and 56 cell/µl for MRC-5, ARPE-19 and HMEC-1 respectively) were observed when a concentration of 2500 cell/µl was processed by the modified CL buffer. Therefore, optimized cell concentration that can be fully lysed by the CL buffer was 500 cell/µl for three cell types.

Table 3.5 Cell lysis capacity of the modified CL buffer

Cell concentration (cell/µl)	No. of unlysed cells (cell/µl)		
	HMEC-1	ARPE-19	MRC-5
2500	56	36	192
500	0	0	0
100	0	0	0
20	0	0	0
4	0	0	0

3.2.3 RNA yields of RNA sample preparations with or without extraction

Finally, the performance of the modified CL buffer was compared with the MagaZorb RNA extraction kit in terms of RNA purification. Both RNA purification methods gave a linear relationship for serially diluted samples ($R^2 \geq 0.97$) (Figure 3.8). For the comparison in MRC-5 and ARPE-19, there was no significant difference in RNA yields between two methods ($P > 0.05$) (Table 3.6). However, a significant difference between the two methods was observed in yielding RNA from HMEC-1 ($P < 0.05$), suggesting that the modified CL buffer offered a higher degree of HCMV RNA for RT-qPCR detection in HMEC-1.

Table 3.6 Comparison between modified CL buffer and RNA extraction for RNA yields from viral cultivation (log_{10}IU/well)

HCMV input	CL buffer/RNA extraction		
	MRC-5	ARPE-19	HMEC-1*
4	5.01/5.01	5.17/4.68	5.05/4.91
3	3.58/3.50	4.53/4.32	4.57/4.20
2	2.61/2.36	2.82/2.73	2.90/2.69
1	1.26/1.12	2.17/2.08	2.09/1.73
0.5	-	1.43/1.42	1.48/1.51
0	0.54/0.00	-	-

* Significant difference ($P < 0.05$)

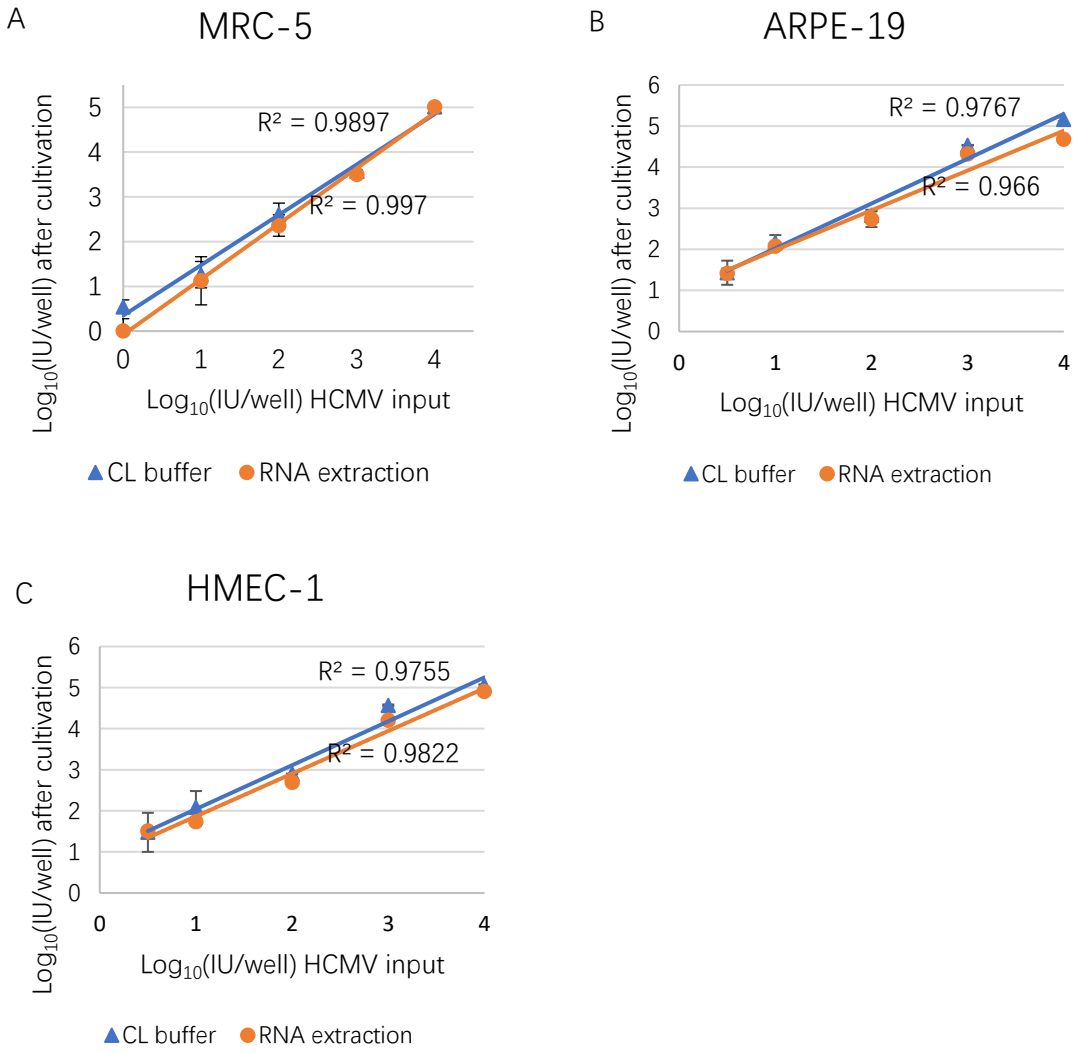


Figure 3.8 Comparison of the modified cell lysis (CL) buffer and RNA extraction methods. A serial dilution of VR1814 from 10^4 to 1 PFU/well in maintenance medium was used to inoculate (A) MRC-5, (B) ARPE-19, and (C) HMEC-1 cells in two 96-well plates. After 20-hour incubation, two plates were processed by MagaZorb® Total RNA Mini-Prep Kits and 100 µl of modified CL buffer respectively, followed by RT-qPCR analysis in the same RT-qPCR run (n=3).

3.3 Comparison of the RT-qPCR assay with the immunostaining assay

The ultimate goal of this study was using the RT-qPCR assay to replace the immunostaining assay in neutralization studies.

An excellent correlation (R^2=0.97) between the two assays was observed on assessing VR1814 infectivity from 25 to 2000 PFU/well viral input in MRC-5 (Figure 3.9). The linear trendline of plotted points slightly shifted to the immunostaining side, indicating that RT-qPCR might be more sensitive than immunostaining in quantifying infectious HCMV virions.

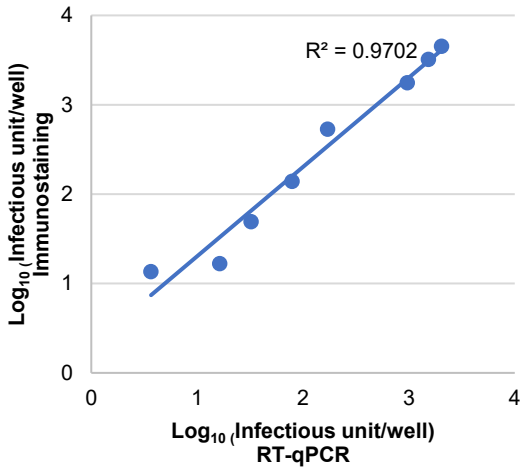


Figure 3.9 Correlation between the RT-qPCR and immunostaining assay in determination of infectious HCMV virions
A series of diluted VR1814 were used to inoculate MRC-5 cells in two 96-well plates. After 24-hour incubation, two plates were analyzed by RT-qPCR and immunostaining respectively. Data from the immunostaining was plotted on the y-axis against data from the RT-qPCR on the x-axis, and a regression line was drawn (n=6).

The sensitivity of the RT-qPCR assay can be as low as 1 PFU/well in the VR1814 infectivity assessment in MRC-5 (Table 3.7), although the results of 1 PFU/well were not as accurate as 10 and 20 PFU/well. In contrast, the immunostaining assay was only able to detect 20 PFU/well in all six runs, indicating that RT-qPCR was 10 times more sensitive than immunostaining in measuring HCMV infectivity after viral culture.

The RT-qPCR-based assay had no false positives in ten replicates of negative controls, while the immunostaining yielded three false-positive results in negative controls. After a manual check under a light microscope, the stained spots of immunostaining in negative control wells were false positive caused by crystalline particle backgrounds which might come from the staining process.

Overall, the RT-qPCR-based assay showed higher sensitivity and specificity than the immunostaining-based assay in quantification of HCMV infectivity after viral culture.

Table 3.7 Comparison of sensitivity and specificity of RT-qPCR-based and immunostaining-based assays

HCMV input (PFU/well)	RT-qPCR		Immunostaining	
	Mean±SD (IU/well)	No. pos[a]	Mean±SD (viral particles/well)	No. pos[a]
20	22.47±3.58	6/6	4±2.76	6/6
10	10.1±5.99	6/6	0.67±1.03	2/6
1	6.55±5.1	6/6	0	0/6
Neg controls	-	0/10	0.4±0.7	3/10

[a] Number of positive results out of six replicates

Both assays were further applied to neutralization measurement of HIG Hizentra against VR1814 to investigate the concordance between two assays. The RT-qPCR assay was able to generate neutralization curves with a good fitness of points in MRC-5, ARPE-19 and HMEC-1 cells (R^2>0.98) (Figure 3.10A). The IC50 values determined by the RT-qPCR assay was 393.31 and 8.04 μg/ml in MRC-5 and ARPE-19 respectively, similar to the results of immunostaining assay at 254.36 and 7.23 μg/ml, while the IC50 values in HMEC-1 were 40.3 μg/ml by RT-qPCR and 19.4 μg/ml by immunostaining, where a two-fold difference can be observed (Table 3.8).

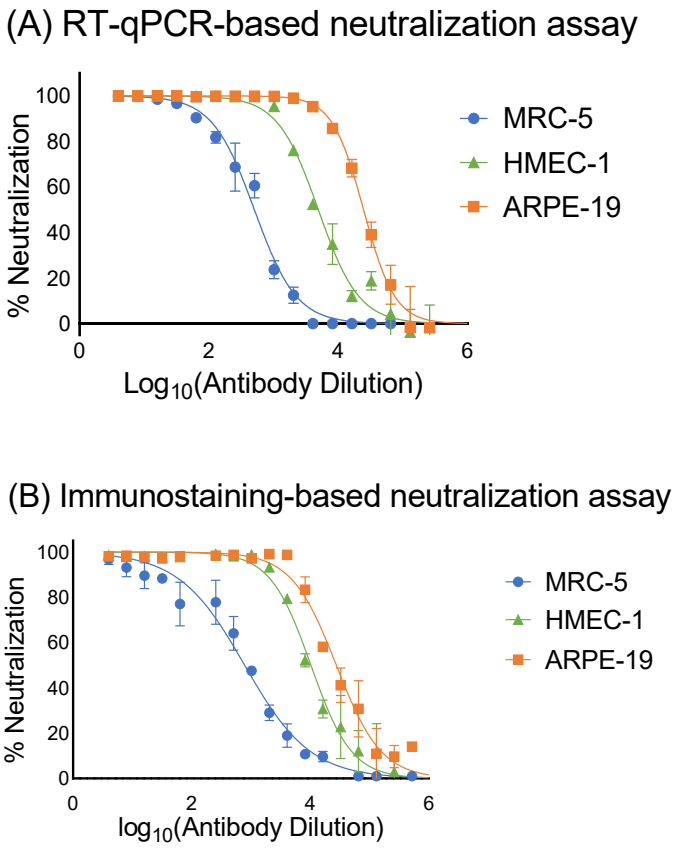


Figure 3.10 Neutralization curves of VR1814 generated by (A) RT-qPCR-based and (B) immunostaining-based neutralization assays
A 2-fold serial dilution of Hizentra from 50 mg/ml to 0.38 μg/ml was incubated with an equal volume of 1000 PFU of VR1814 for 1h and the mixture was used to inoculate MRC-5, ARPE-19 and HMEC-1 cells in 96-well plates. After 24h incubation, the 96-well plates were assessed by (A) RT-qPCR and (B) immunostaining respectively (n=3).

Table 3.8 IC50 values of Hizentra against VR1814 by RT-qPCR-based and immunostaining-based neutralization assays

	RT-qPCR-based neutralization assay		Immunostaining-based neutralization assay	
	Log IC50	IC50 (μg/ml)	Log IC50	IC50 (μg/ml)
MRC-5	2.706	393.31	2.896	254.36
ARPE-19	4.396	8.04	4.442	7.23
HMEC-1	3.696	40.3	4.013	19.4

The concordance between two assays on assessing neutralizing antibody activity against wild-type HCMV from primary clinical specimens was investigated. 162 clinical samples screened as HCMV DNA-positive were neutralized by HIG Cytogam or monoclonal antibodies and detected parallel by RT-qPCR and immunostaining assays. Consistent results from two assays were obtained from 139 out of 162 primary

clinical samples, indicating an excellent concordance at 86% between two assays (Table 3.9). Generally, primary clinical samples with HCMV virions had a higher HCMV DNA load, but no clear correlation can be observed between HCMV infectivity and HCMV viral load in primary clinical samples. 23 out of 162 samples were only positive by RT-qPCR but negative by immunostaining. The difference between the results of the two methods was statistically significant (P<0.05, Fisher's exact test).

Discordant results of 23 samples were listed in Table 3.10 to further investigate whether those RT-qPCR results were false positives. Overall, RT-qPCR positive results of 22 out of 23 samples can be confirmed in different cell lines, different sample types, transmitter-receiver (mother-infant) relationship, or following collections. Only one RT-qPCR positive result of sample M035-1-BRM, which was breast milk from a seropositive mother, could not be confirmed by any of the reasons mentioned above. In the second collection of M035-1-BRM, breast milk of this mother turned out to be HCMV negative, while the urine of her infant started to be HCMV positive. No further sample could be obtained from this mother-infant pair.

In summary, most low positive results obtained by RT-qPCR in primary clinical samples can be considered as true positives expect the sample M035-1-BRM. The discordant results between the two assays were mainly due to the high sensitivity of RT-qPCR.

Table 3.9 The concordance of RT-qPCR-based and immunostaining-based neutralization results on wild-type HCMV

		RT-qPCR	
		Positive	Negative
Immunostaining	Positive	37	0
	Negative	23	102

Table 3.10 The list of samples determined positive by RT-qPCR but negative by immunostaining

No. of samples	Cell line	RT-qPCR (IU/well)			Immunostaining (Spots/well)			
		Cytogam H	Cytogam L	None Cytogam	Cytogam H	Cytogam L	None Cytogam	
T026-4-SAL 24h	MRC-5	1.36	2.25	Neg	Neg	Neg	Neg	Positive by RT-qPCR after 24h and 72h incubation
T026-4-SAL 72h	MRC-5	Neg	Neg	4.22	Neg	Neg	Neg	
B001-URN	ARPE-19	Neg	Neg	2.71	Neg	Neg	Neg	Mother's milk and baby's urine were both positive by RT-qPCR
M002-2-BRM 24h	MRC-5	1.09	1.38	0.95	Neg	Neg	Neg	
M002-2-BRM 72h	MRC-5	0.50	0.81	Neg	Neg	Neg	Neg	
B029-3-SAL	MRC-5	NA	NA	1.37	NA	NA	Neg	The samples from baby B029 kept positive by RT-qPCR for several collections. The sample B029-5-URN contained a large number of culturable HCMV virions that can be determined by both RT-qPCR and immunostaining
	ARPE-19	NA	NA	2.03	NA	NA	Neg	
B029-3-URN	ARPE-19	NA	NA	2.43	NA	NA	Neg	
B029-3-URN 4h	MRC-5	NA	NA	0.53	NA	NA	Neg	
	ARPE-19	NA	NA	3.55	NA	NA	Neg	
B029-5-URN	ARPE-19	NA	NA	2.01	NA	NA	Neg	
	HMEC-1	NA	NA	2.15	NA	NA	Neg	
B029-6-URN	HMEC-1	Neg	0.54	3.87	Neg	Neg	Neg	
M030-5-SAL	MRC-5	Neg	Neg	1.64	Neg	Neg	Neg	
	ARPE-19	Neg	Neg	2.52	Neg	Neg	Neg	
	HMEC-1	Neg	Neg	1.72	Neg	Neg	Neg	
M030-5-BRM	ARPE-19	Neg	Neg	0.63	Neg	Neg	Neg	
	HMEC-1	Neg	Neg	0.62	Neg	Neg	Neg	
M035-1-BRM	ARPE-19	Neg	Neg	0.52	Neg	Neg	Neg	The positive was not able to be confirmed true by other collections or in other cell lines.
T-URN	MRC-5	Neg	Neg	0.81	Neg	Neg	Neg	The sample T-URN was positive in two cell lines by RT-qPCR
	HMEC-1	Neg	Neg	0.52	Neg	Neg	Neg	

Table 3.11 The list of samples determined positive by RT-qPCR but negative by immunostaining (continued)

No. of samples	Cell line	RT-qPCR (IU/well)			Immunostaining (Spots/well)			
		Cytogam H	Cytogam L	None Cytogam	Cytogam H	Cytogam L	None Cytogam	
B038-1-URN	MRC-5	Neg	Neg	0.70	Neg	Neg	Neg	Baby's urine and mother's saliva were both positive by RT-qPCR
M037-1-SAL	ARPE-19	Neg	Neg	1.21	Neg	Neg	Neg	
M053-1-BRM	HMEC-1	Neg	0.56	Neg	Neg	Neg	Neg	The mother's milk were positive by RT-qPCR in two continuous collections
M053-2-BRM	ARPE-19	Neg	Neg	12.65	Neg	Neg	Neg	
M055-1-BRM	ARPE-19	Neg	Neg	0.64	Neg	Neg	Neg	The sample was positive in two cell lines by RT-qPCR
	HMEC-1	Neg	Neg	1.45	Neg	Neg	Neg	
M057-1-BRM	ARPE-19	2.19	1.06	0.68	Neg	Neg	Neg	M057-1-BRM had infectious HCMV in MRC-5 that can be detected by both RT-qPCR and immunostaining
B058-1-URN	ARPE-19	2.94	2.66	2.61	Neg	Neg	Neg	
M061-2-BRM	ARPE-19	0.36	Neg	Neg	Neg	Neg	Neg	M061-2-BRM had infectious HCMV in MRC-5 that can be detected by both RT-qPCR and immunostaining
	HMEC-1	0.39	1.47	2.09	Neg	Neg	Neg	
B056-3-URN	MRC-5	5.33	5.51	8.85	Neg	Neg	0.60	B056-3-SAL had infectious HCMV in MRC-5 that can be detected by both RT-qPCR and immunostaining

3.3.1 Antibody neutralization of wild-type HCMV from clinical specimens

To detect if wild-type HCMV was resistant to neutralizing activities of human immunoglobulin and monoclonal antibodies, a urine sample from an 8-month-old baby with a qPCR Ct value at 29.1 and a saliva sample from a 4-month-old baby with a qPCR Ct value at 14.81 were selected to conduct the full panel neutralization test in MRC-5 and analyzed by both assays. HIG Cytogam and monoclonal antibodies anti-gB 6B4, anti-gB 2B11, anti-gH 11B12 and anti-pentamer 8l21 were included in the antibody panel (Table 3.11). The neutralization results were compared with lab strain VR1814 and shown in Figure 3.11 and Figure 3.12 respectively.

At least 90% of VR1814 were neutralized by the antibody panel except 8l21 in both cases. 8I21 was incapable of neutralizing either wild-type HCMV from clinical specimens or VR1814. Urine HCMV showed high resistance to neutralization induced by HIG or monoclonal antibodies. Only anti-gB antibody 6B4 demonstrated partial neutralizing activities (41.1% by immunostaining and 34.4% by RT-qPCR) against urine HCMV virions at a concentration of 50 μg/ml (Figure 3.11). In contrast, saliva HCMV was more sensitive to neutralizing antibodies (Figure 3.12). HIG Cytogam, anti-gB antibody 6B4, and anti-gH antibody 11B12 showed varying degrees of neutralizing activities against saliva HCMV.

For the comparison between RT-qPCR-based and immunostaining-based neutralization assay, some discrepancies in results can be observed. For example, the RT-qPCR assay demonstrated a 20.5% of Cytogam neutralization at the concentration of 1280 μg/ml, while the immunostaining assay only showed the neutralization rate at 7.7%. In addition, the RT-qPCR assay indicates that high concentrations of Cytogam and 11B12 had relatively potent neutralizing activities which can neutralize 65.9% and 48.4% of saliva HCMV virions, but the immunostaining assay showed that both antibody neutralizations were low (19% and 10.31% respectively).

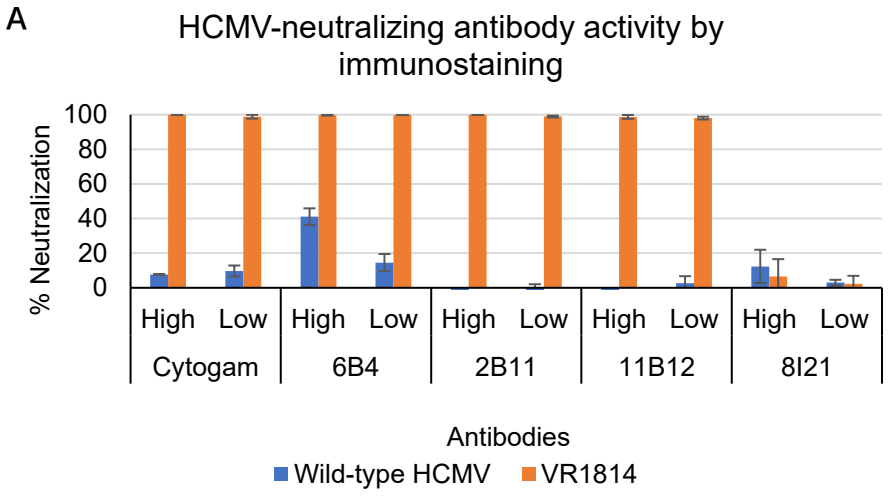
However, both assays were in agreement with most neutralization results and gave a similar pattern of neutralization resistance in HCMV in clinical specimens.

In summary, although there were discrepancies in two assays, both of them showed neutralization resistance was widely present in these HCMV virions from primary clinical samples and indicated anti-gB antibody 6B4 was the most potent neutralizing antibody for wild-type HCMV in primary clinical samples.

Table 3.12 Information on the panel of antibodies*

MAb	Ig isotype	IC90 (μg/ml) for VR1814 in MRC-5	Specificity	Expected neutralization in cell lines
Cytogam	-	-	-	MRC-5, ARPE-19, HMEC-1
6B4	IgG1, κ	1	gB site 1	MRC-5, ARPE-19, HMEC-1
2B11	IgG3, λ	0.75	gB site 3	MRC-5, ARPE-19, HMEC-1
11B12	IgG1, κ	3.5	gH site 2	MRC-5, ARPE-19, HMEC-1
8I21	IgG1, κ	> 10 μg/ml	gH/gL/UL128/130 site 7	ARPE-19, HMEC-1

*Table modified from Annalisa Macagno *et al.* in the Journal of Virology in 2009



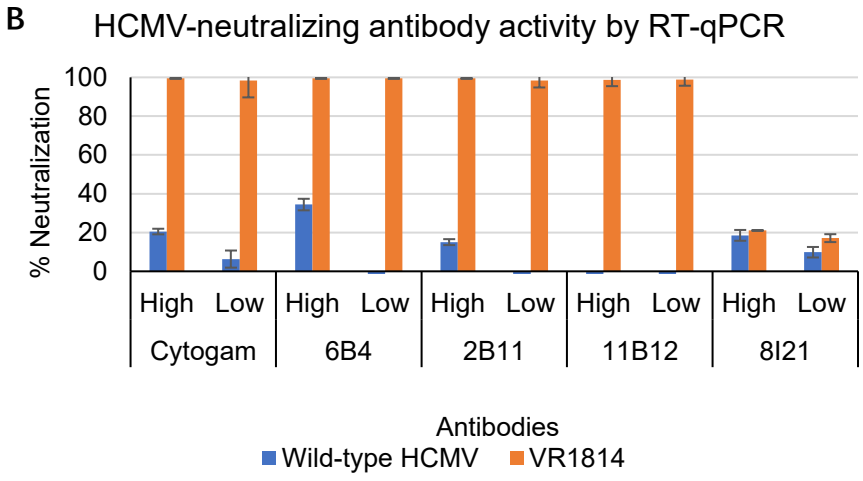


Figure 3.11 Percentages of antibody neutralization of HCMV from a urine sample and VR1814 detected by (A) immunostaining-based and (B) RT-qPCR-based neutralization assays
Human immunoglobulin Cytogam at 1280 and 640 μg/ml, anti-gB mAb 6B4 at 50 and 10 μg/ml, anti-gB mAb 2B11 at 50 and 7.5 μg/ml, anti-gH mAb 11B12 at 50 and 35 μg/ml, and anti-pentamer mAb 8I21 at 50 and 25 μg/ml were incubated with equal volume of urine for 1h and the mixture was used to inoculate MRC-5 cells in 96-well plates. After 24h incubation, the 96-well plates were assessed by (A) RT-qPCR and (B) immunostaining respectively (n=3).

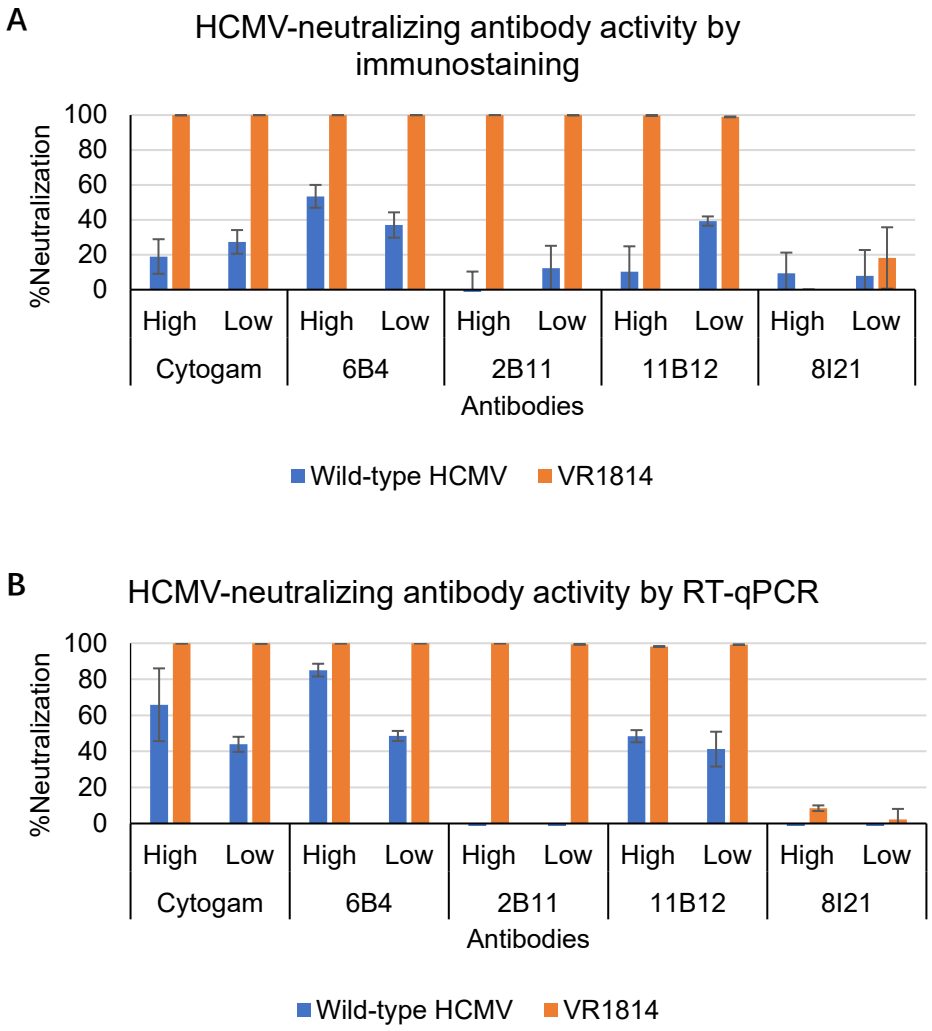


Figure 3.12 Percentages of antibody neutralization of HCMV from a saliva sample and VR1814 detected by (A) immunostaining-based and (B) RT-qPCR-based neutralization assays
Human immunoglobulin Cytogam at 2100 and 1280 μg/ml, anti-gB mAb 6B4 at 50 and 10 μg/ml, anti-gB mAb 2B11 at 50 and 7.5 μg/ml, anti-gH mAb 11B12 at 50 and 35 μg/ml, and anti-pentamer mAb 8I21 at 50 and 25 μg/ml were incubated with equal volume of urine for 1h and the mixture was used to inoculate MRC-5 cells in 96-well plates. After 24h incubation, the 96-well plates were assessed by (A) RT-qPCR and (B) immunostaining respectively (n=3).

Due to the limited sample volume and low HCMV load, the rest of HCMV DNA-positive primary clinical samples (35 out of 37) were only neutralized by HIG Cytogam at a high concentration of 1280 μg/ml and a low concentration of 640 μg/ml.

The neutralization rates of two concentrations of Cytogam could only be roughly calculated in 10 samples. Limited HCMV virions (about 10 viral particles per sample) in those primary clinical samples resulted in high variability. Some contrary results could be observed. For example, in samples M053-3-BRM, B058-1-URN and M068-1-BRM, the neutralization rates of Cytogam were below zero, suggesting that HCMV

with antibodies yielded more infectious virions than without antibodies (Table 3.12). In contrast, other samples showed that Cytogam inhibited HCMV infectivity to some degree, providing neutralization rates approximately from 20 to 50%. Other contrary results can be observed in M053-1-BRM, M057-1-BRM, and B056-2-URN, where higher Cytogam levels showed higher neutralizing activity, while M068-1-BRM in RT-qPCR and B058-1-URN in immunostaining indicated the opposite (Table 3.13).

However, the concentrations of Cytogam (1280 and 640 μg/ml) used in this test were able to neutralize almost all VR1814 virions (≥98%). Although there was inconsistency in RT-qPCR and immunostaining results, neutralizing activity of Cytogam was less than 50% in most cases, significantly lower than its neutralizing activity in VR1814 (≥98%). Therefore, HCMV neutralization resistance may present widely in primary clinical samples.

Table 3.13 Neutralizing activity of Cytogam against wild-type HCMV in primary clinical samples by RT-qPCR in MRC-5

Clinical samples	Wild-type HCMV			VR1814	
	%Neutralization Cytogam H	%Neutralization Cytogam L	None Cytogam (IU/well)	%Neutralization Cytogam H	%Neutralization Cytogam L
M053-1-BRM	28.74	13.48	8.18	99	99
M053-3-BRM	7.54	-[a]	6.41	99	98
M055-1-BRM	32.36	10.69	27.05	99	98
B056-2-URN	50.73	27.6	11.94	99	99
B056-3-URN	39.77	20.03	32.44	99	98
B056-4-URN	55.61	33.16	60.44	99	99
M057-1-BRM	51.96	1.82	4.84	99	99
B058-1-URN	-[a]	-[a]	7.11	99	99
M068-1-BRM	0	34.64	12.02	99	98
B069-3-URN	40.7	15.12	7.32	99	99

[a] the value of %Neutralization is a negative number

Table 3.14 Neutralizing activity of Cytogam against wild-type HCMV in primary clinical samples by immunostaining in MRC-5

Clinical samples	Wild-type HCMV			VR1814	
	%Neutralization Cytogam H	%Neutralization Cytogam L	None Cytogam (Spots/well)	%Neutralization Cytogam H	%Neutralization Cytogam L
M053-1-BRM	51.61	43.55	6.2	100	100
M053-3-BRM	69.39	36.73	4.9	100	100
M055-1-BRM	28.57	61.04	7.7	100	99
B056-2-URN	13.79	3.45	5.8	100	100
B056-3-URN	100	100	0.6	100	100
B056-4-URN	25.4	35.8	6.7	100	99
M057-1-BRM	52.63	0	9.5	100	100
B058-1-URN	-[a]	100	0.2	100	100
M068-1-BRM	0.75	-[a]	6.65	100	99
B069-3-URN	NA	NA	NA	100	100

[a] the value of %Neutralization is a negative number
NA Not available for immunostaining-based neutralization tests

Chapter 4 Discussion

4.1 Evaluation of the one-step RT-qPCR assay

The goal of the project was to select an alternate HCMV neutralization assay with high accuracy, high precision and less labor input to replace the immunostaining-based neutralization assay. After reviewing existing scientific literature regarding novel nucleic acid-based neutralization methods, preliminary analysis was carried out on several reported RT-qPCR methods for HCMV detection. Bergallo *et al*. reported an RT-qPCR assay with a pair of primers targeting a 116 bp RNA segment on IE1 gene transcription (98), but cross-reactivity with viral DNA could be observed and RNA isolation must be conducted before RT-qPCR analysis by using DNase. Therefore, this reported method was excluded due to the tedious procedure. Another pair of primers for UL89 including an exon-exon junction described by Wang *et al*. was also preliminary analyzed (97). The primers in this method were specific to HCMV RNA only, but UL89 is referred to as an early-late gene. Considering the slow replication cycle of wild-type HCMV and the need for a rapid HCMV detection in this study, the UL89 method was excluded as well.

A one-step RT-qPCR assay based on SYBR green system capable of detecting the presence of HCMV mRNA aroused interest (97). The RT-qPCR-based assay has shown many advantages, including early detection at 6 h.p.i., high sensitivity and the capacity to process large numbers of samples at a time. However, the RT-qPCR assay has not been thoroughly validated in the literature, which leads to the initiative of this study, the evaluation of the RT-qPCR assay. Furthermore, as qPCR is the most common molecular method for HCMV detection, this study also attempted to apply our laboratory-developed qPCR assay used routinely to HCMV neutralization studies and compare it with the RT-qPCR-based assay.

The results of RT-qPCR analysis are the number of threshold cycles, usually called the Ct value. Most real-time RT-PCR assessments converted Ct value into genome copy numbers to estimate RNA quantity using standard curves. However, it is difficult to interpret the meaning of RNA copy numbers of HCMV in a neutralization test that aims to measure viral infectivity. To fit the goal of applying the RT-qPCR to a neutralization assay, the HCMV RNA standard curve using in this thesis was calibrated with a serial dilution of infectious HCMV titrated by a plaque assay. Therefore, HCMV RNA against Ct values of RT-qPCR can be directly converted into infectious HCMV titers (infectious units/reaction) with good confidence ($R^2 > 0.99$), indicating the reliability of using Ct

value to predict HCMV infectivity, thereby bridging the gap between RNA copy numbers and viral infectivity.

The RT-qPCR assay was a SYBR green-based method, which is cheaper and faster than a probe-based method, but it is difficult to distinguish true positives (specific SYBR green dye binding) from false positives (nonspecific SYBR green dye binding). The Tm range of true HCMV amplicon (76.8 to 78.8°C) obtained from positive control was first established by the RT-qPCR assay to solve the nonspecific binding problem. Additionally, a Ct value of 34 was defined as the cutoff value for RT-qPCR based on the equation $Infectious\ units = \frac{33.977 - Ct\ value}{3.3864}$ generated by the linearity test to fit the purpose of minimizing false-positive results. With effort, the SYBR green-based RT-qPCR became a promising assay to provide reliable results.

High sensitivity is one of the biggest advantages of molecular methods such as RT-qPCR, but the sensitivity of this RT-qPCR assay has not been reported. This thesis demonstrated that the RT-qPCR was able to detect HCMV as low as 0.6 IU/reaction, which is believed to be sensitive enough for neutralization studies.

In addition to sensitivity, specificity is another important factor for RT-qPCR. In many published qPCR assays for HCMV detection, BK virus (BKV) and Epstein-Barr virus (EBV) are the most common viruses needing to be confirmed as not having cross-reactivity with PCR primers, as BKV and EBV are opportunistic pathogens as common as HCMV among immunosuppressed individuals (132–134). Clinical specimens involved in this study might contain BKV and EBV particles, but the cell lines (MRC-5, ARPE-19 and HMEC-1) used here are not normal target cells for BKV or EBV (135,136). The short incubation time (20 or 48 hours) also prevents other viruses from proliferating in those cells (78). Therefore, this study did not investigate cross-reactivity with other viruses.

Even though the primers designed in this assay spanned an exon-exon splice site, there was still concern that HCMV DNA may cross-react with the primers and compromise the specificity of this assay. The validation in this thesis confirmed that the RT-qPCR assay did not yield any positive results for the five RNA-free HCMV DNA samples, indicating that there is no cross-reactivity between viral RNA and DNA.

This study demonstrated that the RT-qPCR assay has high intra- and inter-run precision for high and medium levels of HCMV viral load. With the decrease of HCMV load in samples, an increase in coefficient of variation and standard deviation were

observed, which is understandable as the degree of imprecision is expected near the limit of detection (137). There is no universal agreement on acceptance criteria for precision, but most published guidelines recommended a CV below 20% or below 35% as acceptable for precision of PCR assays (138–140). Therefore, CVs for low positive samples giving a value at around 9% for intra-assay precision and a value at around 15% for inter-assay precision of the RT-qPCR assay were considered acceptable.

RNA and DNA growth kinetics demonstrated that sufficient VR1814 RNA has been yielded at 20 h.p.i. in MRC-5, ARPE-19, and HMEC-1, while there was no significant DNA accumulation within the first 50 hours after inoculation. A possible reason for this difference is that the primers used in RT-qPCR are designed to target an RNA fragment from IE1 gene, which is the first HCMV gene starting transcription after viral infection (20). In contrast, viral genome replication was initiated after IE gene expression (17). Therefore, the RT-qPCR assay could monitor the earliest signal of viral proliferation while the qPCR assay could not. Similar findings were reported that AD169, another laboratory-adapted HCMV strain similar to VR1814, began viral DNA synthesis after 24 h.p.i. and reached a plateau of accumulation at 72 to 96 h.p.i. (28). Additionally, the magnitude of DNA accumulation is also not comparable to RNA accumulation. A 5-fold increase in the amount of AD169 DNA was reported to be observed after 3 to 4 days of viral infection (28), dramatically slower and quantitatively lower than the changes in RNA loads (1000-fold in 20 hours). Therefore, HCMV RNA is a better indicator than DNA for indicating viral propagation in terms of detection time and magnitude.

That paper originally describing the RT-qPCR assay also examined HCMV RNA kinetics and demonstrated that sufficient RNA is yielded at 6 h.p.i.. A possible reason for the difference in timepoints between their paper and this thesis is that the HCMV strain they used was AD169, a highly mutated strain that can only be cultivated in fibroblasts. In contrast, VR1814 used in this thesis was a clinical isolate with low passages that can still inoculate a wide range of cell types including epithelial and endothelial cells. Therefore, AD169 has developed a greater growing capacity than VR1814 *in vitro* and is able to provide a large amount of RNA right after viral infection.

Due to the observation of the growth rate difference between AD169 and VR1814, a similar difference between VR1814 and wild-type HCMV was expected. However, surprisingly, RT-qPCR assessment at 20 h.p.i. was also suitable for measuring the

infectivity of HCMV in primary clinical samples. Many studies suggested that HCMV from clinical specimens required about two weeks to spread and form potential cytopathic effect (CPE) in tissue culture, significantly longer than laboratory-adapted strains (two days to one week) (141,142). Little is known about the time required for each replication stage for wild-type HCMV strains, but the reasons for RT-qPCR assessment at 20 h.p.i. applicable to both wild-type and laboratory-adapted strains could be that (1) mutations have not been induced much in low-passage strains such as VR1814, which leads to a similar growth rate with wild-type strains; (2) the growth rate difference between low-passage and wild-type strains may not be demonstrable at the early stage of viral replication.

Some reported immunostaining assays used the same 24h incubation time for both lab strains and clinical specimens (143,144), while others extended to 72 hours for both lab and wild-type strains to obtain strong staining spots and minimize false-negative results (42,145). The target of the immunostaining assay was a protein produced by IE genes, which is an early-stage product in HCMV replication cycles. A study showed that 48h incubation was the best time point for IE protein-based immunostaining to balance rapid HCMV detection purposes and sensitivity (93%) for clinical specimens (141). Therefore, the 48h incubation was used for the immunostaining assay in this study. To ensure the two assays were comparable and had fewer false-negative results, RT-qPCR assay was also performed after 48h of incubation since there was no statistical difference between 20h and 48h for RT-qPCR assessing wild-type HCMV infectivity.

In summary, the RT-qPCR assay used in this thesis has demonstrated high sensitivity, specificity and precision. The RT-qPCR assessment was performed at 20 h.p.i. for VR1814 and at 48 h.p.i. for wild-type HCMV in primary clinical samples.

4.2 Optimization of the RT-qPCR-ready cell lysates for RT-qPCR

Nucleic acid extraction is an unavoidable step to obtain purified RNA for classic RT-qPCR analysis, but it is rate-limiting for large scale experiments such as neutralization assays that have to process RNA extraction on over 60 wells of cell monolayers at a time. Commercial reagents can generate cell lysates which can be directly analyzed by RT-qPCR, but the high cost prevents them from broad use. A recently published paper described a method for production of in-house cell lysis buffer and successfully applied the buffer to lysing MRC-5 and ARPE-19 cells (112).

Nevertheless, strong PCR inhibition was observed when the CL buffer was used in our study and the main inhibitors were found in the cell lysates. Possible reasons for our failure to repeat the published experiment could be: (1) the incubation time in this study (20 or 48 hours) was longer than theirs (6 hours). Cells kept proliferating in the extended incubation time and might exceed the maximum number of cells the CL buffer can process. (2) our study used clinical specimens to inoculate cells rather than the purified viruses in their experiment. Many components from clinical samples contain potential inhibitors (e.g. urea in urine or enzyme in saliva) that can impact PCR amplification, leading to the failure of application of CL buffer (146,147).

In order to simplify RNA purification steps, the cell lysis procedure was modified. The optimized CL buffer could yield more HCMV RNA for RT-qPCR analysis than commercial RNA extraction kits. This finding was consistent with some studies suggesting that nucleic acid extraction causes loss of material (107). Additionally, 100µl of the modified CL buffer could lyse up to 50,000 cells, dramatically exceeding the cell number (about 30,000 cells/well) in 96-well plates, which allows adequate exposure of viral RNA from the cells in each well.

In conclusion, the modified cell lysis procedure is able to yield HCMV RNA from cell monolayers for downstream RT-qPCR analysis without causing amplification inhibition. This is a simple and efficient RNA purification method superior to nucleic acid extraction.

4.3 Comparison of the RT-qPCR assay with the immunostaining assay

The RT-qPCR assay was 10 times more sensitive than the immunostaining assay on measuring the infectivity of cultivated HCMV virions. Similar results were also reported by Skog *et al.* who found that qPCR was more sensitive than immunostaining with a limit of detection of less than 20 virus particles (148). Wechsler *et al.* demonstrated that the sensitivity of immunostaining was related to the expression intensity of target antigens (149). Early staining may compromise the sensitivity due to deficient protein synthesis, but once incubation time was extended to 3 days, the limit of detection of immunostaining could be increased to around 0.3 PFU/ml in their study (149,150).

RT-qPCR also showed superior specificity to immunostaining. This is not surprising since immunostaining is known as a method with high sensitivity but

relatively low specificity (151). Although indirect staining could reduce nonspecific binding and amplify signals, an overestimation of positive results was still commonly reported when using immunostaining-based assays (148,150,152).

In neutralization studies of HCMV in clinical samples, a concentration of HCMV virions at around 200 PFU/well would be highly valued but hard to obtain. Most clinical samples with infectious HCMV virions gave a viral concentration at around 10 to 50 PFU/well (43). Therefore, the ability to accurately detecting HCMV particles at low levels was the most important feature in this study, and RT-qPCR demonstrated higher sensitivity and specificity to meet the need.

The RT-qPCR-based neutralization assay was able to generate neutralization curves in MRC-5, ARPE-19, and HMEC-1. An excellent fitness of points (R^2>0.98) indicates that the curves were reasonable and reliable. IC50 values from the two assays were highly consistent in ARPE-19 and MRC-5, but two-fold different in HMEC-1.

IC50 is an important parameter used to study drug sensitivity and neutralization resistance, but complex experimental and data-analysis processes make IC50 less reliable and assay-specific (153–155). A guideline established by the International Society for Influenza and other Respiratory Virus Diseases indicated that a 2 to 3-fold difference in IC50 was common for drug-virus inhibition assays (156). Degraeve *et al.* reported an interlaboratory variability of IC50 up to 88% (157). Kalliokoski *et al.* suggested that IC50 values were not comparable between assays and using an average of all available IC50 data would be more accurate and applicable than use of a single IC50 value (158). Therefore, the 2-fold difference between RT-qPCR-based and immunostaining-based neutralization assays in IC50 values obtained in HMEC-1 would still be considered acceptable. Overall, both assays have shown the capacity to generate reliable neutralization curves and reasonable IC50 values.

Both RT-qPCR-based and immunostaining-based neutralization assays were used to evaluate the neutralizing activity of HIG and a monoclonal antibody panel. A good concordance was obtained between two assays. Most discrepancies (22 out of 23) in the comparison were caused by the high sensitivity of the RT-qPCR assay. Only one RT-qPCR positive result for breast milk from a mother M035 was not confirmed. It could be a false negative, but since the baby's urine and saliva were HCMV DNA positive in the second collection, it is more likely that HCMV infectious particles were being shed from mother's milk, leading to HCMV infection in the baby followed by

viral clearance in mother's breast milk. However, samples were only able to be collected twice in this mother-infant pair, so there was no further data to confirm the hypothesis.

In conclusion, this study has shown an excellent agreement between RT-qPCR-based and immunostaining-based assays and confirmed that RT-qPCR is more sensitive than immunostaining.

4.4 HCMV neutralization resistance in clinical specimens

HCMV neutralization resistance in some mutated strains was observed and reported at the beginning of the 21st century (143,144). In 2017, Cui *et al*. reported that HCMV from ten urine samples were resistant to neutralization with HIG and monoclonal antibodies, indicating neutralization resistance was not limited to mutated HCMV strains but widely present in wild-type HCMV virions from urine tested as a primary sample (43).

In this study, compared with the high neutralization rates of VR1814, wild-type HCMV virions obtained from urine and saliva have shown a high resistance to neutralizing antibodies, which is in agreement with Cui *et al*.'s finding. Other wild-type HCMV particles in primary clinical samples were only neutralized with HIG due to limited sample volume, but similar neutralization resistance can also be observed by both RT-qPCR and immunostaining assays, indicating a wide presence of HCMV neutralization resistance against current in-use neutralizing antibodies.

In the neutralization tests conducted with full monoclonal antibody panel, it is not surprising that 8I21 was not able to neutralize either VR1814 or wild-type HCMV in MRC-5. 8I21 was the antibody binding to gH/gL/UL128/130, which forms parts of the pentamer complex gH/gL/UL128-131 of HCMV (131). The pentamer complex is an important component required for HCMV entry to epithelial or endothelial cells, but not to fibroblasts (15). Therefore, 8I21 was incapable of preventing either lab strains or wild-type strains of HCMV from spreading in MRC-5.

6B4 and 2B11 were both monoclonal antibodies specific to gB but binding to different antigenic sites. The IC90 values of 6B4 and 2B11 for VR1814 were similar (1 and 0.75 μg/ml respectively) in MRC-5, but 6B4 was able to provide partial neutralizing activity against wild-type HCMV in primary urine and saliva samples while 2B11 was not. The mechanism of the difference in their neutralizing activity is unknown, but the

distinct antigenic sites they recognized may be a key to it.

Cytogam and 11B12 were also observed effective to partially neutralize wild-type HCMV in saliva. Cytogam was purified human IgG derived from adult human plasma pools with a high titer of IgG antibodies against HCMV. Therefore, it is possible that Cytogam may contain antibodies able to bind to accessible epitope(s), thereby inhibiting HCMV infectivity. However, Cui *et al*. indicated that Cytogam failed to neutralize wild-type HCMV in primary urine samples (43). Possible reasons for this discrepancy include: (1) the neutralizing activity of Cytogam is strain-specific; (2) Cytogam was pooled IgG selected based on anti-HCMV titers but not neutralizing activity. Falk *et al*. reported that there was no correlation between anti-HCMV titers and neutralizing activity (159), leading to the lot-to-lot variation of Cytogam on neutralization. Therefore, the Cytogam lot used in Cui *et al*.'s study may have low neutralization effect. 11B12 was a monoclonal antibody binding to an epitope in gH. gH is a necessary glycoprotein for viral fusion and forms the trimeric and pentameric complex which are important to viral entry (12). Therefore, 11B12 is likely to have potential neutralizing capacity against wild-type HCMV in primary clinical samples, but since its partial neutralization was only observed in one primary saliva sample, further studies regarding its neutralizing activity still need to be conducted.

One interesting finding in this neutralization study is that neutralizing antibodies effective to wild-type HCMV strains in primary saliva samples were higher in number and magnitude than those in primary urine samples. Some next generation sequencing studies have shown that genomic difference presented in HCMV strains from the same individual but different compartments (160,161). Nelson *et al*. reported that compared with the placebo group, HCMV viral load reduced in saliva of gB vaccine recipients but not in urine (162). The mechanism of those differences is unclear, but it is possible that HCMV strains may evolve separately in intrahost compartments due to the natural selection of different organs, leading to distinct neutralization sensitivity between HCMV urine and saliva populations.

Immunostaining and RT-qPCR were consistent with indicating that 6B4 was the most promising neutralizing antibody for wild-type HCMV, and Cytogam as well as 11B12 were also capable of neutralizing HCMV from saliva. However, discrepancies between the results of the two assays could also be observed. Since only two clinical specimens were included in the neutralization tests with the full antibody panel, the data

was not sufficient to conclude which assay was more accurate. However, it was reasonable to believe that immunostaining was less accurate as previous studies have shown immunostaining had higher variability and its results of lower concentration of antibodies with higher neutralizing activity also conflicted with knowledge.

4.5 Summary of findings

In this study, An RT-qPCR-based neutralization assay combined with RT-qPCR-ready cell lysates was evaluated and optimized. The RT-qPCR assay was superior to immunostaining assay in sensitivity, specificity, and precision for HCMV neutralization assessment. HCMV neutralization resistance was observed in wild-type strains from saliva, urine, and breast milk as primary clinical samples.

The sensitivity of RT-qPCR assay can be as low as 0.6 IU/reaction with a dynamic range from 1 to 1×10^4 PFU/reaction. The assay for HCMV RNA detection had no cross-reactivity with HCMV DNA and had high inter- as well as intra-assay precision. RT-qPCR analysis can be conducted as early as 20h post-infection for laboratory-adapted and wild-type HCMV strains. The in-house cell lysis buffer was optimized to eliminate inhibitors in cell lysates by simply adding vortex and centrifugation steps after cell lysing, and more detectable HCMV RNA can be offered by the optimized cell lysis buffer than by MagaZorb RNA extraction kits.

The results of RT-qPCR assay were comparable to immunostaining assay for HCMV infectivity analysis and HCMV neutralization tests, and high agreement can be observed between two assays. The RT-qPCR assay for HCMV neutralization was 10 times higher the than immunostaining assay. No false-positive RT-qPCR result was observed in neutralization tests for laboratory-adapted and wild-type HCMV strains using primary clinical samples.

High neutralization resistance was observed for HCMV in saliva, urine, and breast milk specimens in MRC-5. Anti-gB monoclonal antibody 6B4 has some neutralizing activity against HCMV in saliva and urine, but less than the activity against laboratory-adapted strains. Human immunoglobulin Cytogam and anti-gH monoclonal antibody 11B12 was able to partially neutralize HCMV from saliva.

This study indicates the RT-qPCR assay can be an alternative method for HCMV neutralization with high sensitivity, high specificity as well as excellent precision. Neutralization resistance is widely present in wild-type HCMV virions from clinical

specimens in fibroblasts.

4.6 Limitations of this study

This study has some limitations. The first limitation is the limited sample source. The original plan for recruitment is to include primary infected pregnant women, congenitally infected infants, breastfeeding mother-infant pairs, and transplant recipients. However, only one congenital HCMV infection case was recruited until this thesis was completed. The majority of transplant recipients were HCMV-negative due to antiviral prophylaxis medication after transplantation and intensive HCMV monitoring. Therefore, most HCMV positive samples were collected from infants infected by HCMV-positive breast milk. The limited sample sources in this thesis may result in potential bias and make the conclusion less applicable to the entire HCMV population.

Moreover, the limited sample volume and low HCMV titers in specimens also prevent this study from drawing a solid conclusion. The volume of saliva and urine collected from breastfed infants ranged from 0 to 500 μl and 2 to 8 ml respectively, which was often insufficient to conduct neutralization tests with the full panel of antibodies. Congenitally infected newborns are more likely to have a high titer of HCMV load, while breastfed infants often acquire infection with low viral titers (43). In this study, most HCMV DNA-positive clinical specimens contained none or only a few infectious virions, which is difficult to perform neutralization tests and to observe potential neutralization resistance. Only two clinical specimens provided relatively high HCMV titers at around 1000 infectious particles per ml, allowing the performance of neutralization tests with a full antibody panel. Therefore, the observation of neutralization resistance in clinical samples and potent neutralizing activity of anti-gB MAb 6B4 in this study could be strain-specific results and may not be an appropriate representative for the whole HCMV population in clinical specimens.

Other limitations of this study include a lack of non-fibroblast data with clinical specimens. Wild-type HCMV virions in clinical samples are easier to replicate in fibroblasts than epithelial and endothelial cells, but the latter two cell types play a more important role in imitating neutralization reaction *in vivo* since epithelial cells function as the first defence line against viral infection and endothelial cells facilitate HCMV latency establishment (62). However, no wild-type HCMV strains in this study could

be cultivated in epithelial or endothelial cells. The main reason for the cultivation difficulty is the low viral titers in clinical samples. Cui *et al*. suggested that a ratio of clinical HCMV infectivity between fibroblasts and epithelial cells was 55:1(43). Based on the number of infectious particles grown in fibroblasts (1000 infectious particles/ml), the estimated number of virions able to grow in epithelial cells would be 3 to 4 per well in 96-well plates, which is insufficient to conduct any neutralization experiments. Many studies suggested that pentamer-specific antibodies may have higher neutralizing activity than gB or gH-specific antibodies in laboratory-adapted strains and clinical isolates (43,131). However, this study failed to demonstrate whether anti-pentamer monoclonal antibody 8I21 could support their findings or not due to a lack of data in epithelial and endothelial cells.

Another limitation is the specificity of the SYBR green-based RT-qPCR assay. A lack of probe in the SYBR green system reduces running costs, but the specificity can also be compromised because SYBR green dye could bind to nonspecific DNA fragments. This problem was partially addressed by performing melting curve analysis and Ct value cut-off in this thesis. The specificity test demonstrated that there was no cross-reactivity between the targeted RNA segment and HCMV DNA sequences. However, the possibility of having false-positive results still can not be fully eliminated, and the implementation of cut-off may impact the sensitivity of the RT-qPCR assay at the low HCMV RNA level.

Chapter 5 Future Directions

The optimized RT-qPCR-based neutralization assay can be used to detect potential neutralization resistance of wild-type HCMV in epithelial and endothelial cells. HCMV is most likely transmitted through body fluids, so epithelial cells act as the first barrier to defend against viruses (53). Endothelial cells played a key role in HCMV spreading *in vivo* and finally disseminating to myeloid lineage cells to develop persistent latency (62). Therefore, it is important to investigate whether monoclonal antibodies were capable of preventing HCMV from transmission among epithelial and endothelial cells and whether antibodies specific binding to the pentameric complex have better neutralizing activity than those binding to gB. Due to the limited sample size, this study did not obtain any clinical specimens with high HCMV titers that can infect epithelial or endothelial cells *in vitro*, but Cui *et al*. showed that wild-type HCMV are able to grow in ARPE-19 cells (43). To my knowledge, there has been no HCMV neutralization resistance study done in endothelial cells, but as a crucial cell type facilitating HCMV transmission *in vivo*, it is worth implementing the RT-qPCR-based neutralization assay on investigation of neutralizing antibody activity against wild-type HCMV in endothelial cells. This implementation would require clinical samples with high HCMV titers, which is more likely to be obtained from congenital HCMV infection cases.

Additionally, for the specimens collected in this study, most saliva samples with promising qPCR Ct values were not culturable *in vitro*, leading to the question about the proportion of infectious HCMV particles versus free HCMV fragments in saliva. Mayer *et al*. divided oral HCMV shedding into viral expansion, transition, and clearance phases according to HCMV DNA levels detected by qPCR, and the high-quantity shedding period was as short as 6 weeks in infants. However, their study failed to demonstrate the infectivity of viral excretion in those phases (163). Furthermore, most HCMV infected children included in this study were 3 to 5-months old; one of the children shedding HCMV virions in saliva was 7-months old. Therefore, it is reasonable to hypothesize that HCMV DNA in saliva may be fragmented cell-free DNA at an early stage of infection followed by production of infectious virions and finally viral clearance. The RT-qPCR assay could be applied to monitor the potential changes in the proportion of HCMV virions in saliva with time and therefore demonstrate the dynamics of infectious HCMV shedding in saliva after infection.

Moreover, some HCMV seropositive mothers in this study were found to have no detectable HCMV in their breast milk, which is in agreement with a review of 26 articles that found 66-96% HCMV positive mothers had detectable HCMV in their breast milk (164). Ehlinger *et al*. observed that the HCMV load in milk was inversely correlated with HCMV-binding IgG avidity (165), leading to the hypothesis that antibodies in seropositive mothers whose breast milk remains HCMV DNA negative may have higher neutralizing activities against HCMV. Lilleri *et al*. conducted a similar study and concluded that there was no significant difference in antibodies between the two subpopulations (166). However, their study divided mothers into transmitters and non-transmitters based on HCMV infection of their infants. It has been shown that low transmission rates in infants can be caused by freezing milk before feeding or feeding with less breast milk but more formula milk (167). Therefore, it is still valuable to investigate any potential difference in neutralizing activities of antibodies from milk-virion-positive mothers, milk-DNA-positive mothers, and milk-negative mothers, and the RT-qPCR assay could be used to evaluate neutralizing antibody activity in milk and screen for antibodies with high neutralizing activities.

As 11B12 showed partial neutralizing activity against saliva HCMV but not against urine HCMV, it would be interesting to further investigate whether the neutralizing activities of monoclonal antibodies were strain-specific and whether salivary gland or urinary system plays a role in HCMV neutralization. Nelson *et al*. suggested that HCMV population in salivary gland may be more neutralization sensitive than HCMV populations in other compartments (162), but this finding was based on an observation on 11 gB vaccinees, which is still a relatively small sample size. Therefore, the RT-qPCR assay can be applied to more wild-type HCMV neutralization tests with anti-gH antibodies to examine whether gH is a glycoprotein widely effective to inhibit wild-type HCMV, and applied to more urine and saliva HCMV neutralization tests to detect if the sensitivity of saliva HCMV to neutralizing antibodies is widely present. Whole genome sequencing can be performed as well to investigate the genetic diversity of HCMV subpopulations in different intrahost organs and uncover potential gene regions responsible for the neutralization sensitivity in saliva.

Another interesting finding in this study is the difference between 6B4 and 2B11 in neutralizing activities. 6B4 and 2B11 bind to different antigenic sites of gB. Their neutralizing activity was very similar in lab strains but significantly different in wild-type HCMV strains. It is reasonable to hypothesize that the epitope(s) 6B4 binds to may be more accessible in wild-type strains than the epitope(s) 2B11binds to. Gerna *et al*. demonstrated that 6B4 and 2B11 recognize distinct sites on gB and 6B4 reacted with gB 69-78 peptide (131,168), which is a antigenic site more conserved among different strains and more functionally important in neutralization (169,170). However, little is known about how gB antigenic sites are involved in neutralization and what is the structural and functional difference between those sites in lab strains and in wild-type strains. Mutations can be induced to block potential epitopes and Western blotting can be used to determine whether bindings of 6B4 and 2B11 are inhibited. Electron microscopy can be used to develop the X-ray structures of gB in order to further investigate possible structural differences of epitopes in wild-type and laboratory-adapted HCMV.

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