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*Article*

# Challenging the conventional interpretation of HCMV seronegativity

**Shelley Waters [1], Silvia Lee [1,2], Ashley Irish [3], Patricia Price [1,*]**

[1] School of Medicine, Curtin Health Innovation Research Institute, Curtin University, Bentley, Australia
[2] Department of Microbiology, Pathwest Laboratory Medicine, Western Australia
[3] Renal Unit, Fiona Stanley Hospital, Murdoch, Western Australia
[*] Correspondence: shelley.waters@postgrad.curtin.edu.au

**Abstract:** The majority of adults in the world (around 83%) carry antibodies reactive with HCMV and are thought to retain inactive or latent infections lifelong. The virus is transmitted via saliva so infection events are likely to be common. Indeed it is hard to imagine a life without exposure to HCMV. From 45 seronegative individuals (13 renal transplant recipients, 32 healthy adults), we present seven cases who had detectable HCMV DNA in their blood and/or saliva, or a CMV-encoded homologue of IL-10 (vIL-10) in their plasma. One case displayed NK cells characteristic of CMV infection, and HCMV DNA became undetectable. In other cases, the infection may persist with seroconversion blocked by vIL-10. Future research should seek mechanisms that can prevent an individual from seroconverting despite a persistent HCMV infection, as HCMV vaccines may not work well in such people.

**Keywords:** Human cytomegalovirus, seronegative, NK cells, viral IL-10

**Abstract:** The majority of adults in the world (around 83%) carry antibodies reactive with HCMV and are thought to retain inactive or latent infections lifelong. The virus is transmitted via saliva so infection events are likely to be common. Indeed it is hard to imagine a life without exposure to HCMV. From 45 seronegative individuals (13 renal transplant recipients, 32 healthy adults), we present seven cases who had detectable HCMV DNA in their blood and/or saliva, or a CMV-encoded homologue of IL-10 (vIL-10) in their plasma. One case displayed NK cells characteristic of CMV infection, and HCMV DNA became undetectable. In other cases, the infection may persist with seroconversion blocked by vIL-10. Future research should seek mechanisms that can prevent an individual from seroconverting despite a persistent HCMV infection, as HCMV vaccines may not work well in such people.

## 1. Introduction

It is often assumed that a person who has antibodies reactive against a specific virus (ie: is defined as "seropositive") has been infected and may retain that virus. The corollary would be that a person who is seronegative has never been infected. It is then a small step to assume that such people have never been exposed to an infectious dose of the virus. Whilst this is logical with novel viruses (eg: Zika or SARS-Cov2), it does not fit the clinical data with respect to human cytomegalovirus (HCMV). The majority of adults in the world (around 83%) carry antibodies reactive with HCMV [1]. Active infections are usually controlled in healthy individuals, but the virus enters a latent or inactive state, and persists with periodic reactivations. In individuals with acquired immune deficiencies, HCMV infections induce diverse but well characterized clinical syndromes, and reactivations are relatively common.

HCMV can be transmitted vertically (transplacentally and through breast-feeding), and via organ transplantation and blood transfusions [2]. However saliva may be the most common route of transmission, as HCMV and murine (M) CMV establish latency in the salivary gland [3, 4]. We have linked the presence of HCMV DNA in saliva with activation of systemic immune responses consistent with a systemic infection [5].

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HCMV may pass to a new host through an oral route [2, 6], though murine studies suggests the intranasal route effectively initiates primary infection [7]. Transmission via saliva is readily demonstrated in nurseries and crèches [8], but is likely in other situations where individuals are close together. This is not restricted to particular populations.

Despite this plethora of opportunities to become infected, some people remain seronegative all their lives. We considered whether this represents a failure to seroconvert when infected or a lifelong failure to become infected. The former is possible as we have identified reports of HCMV DNA in urine from seronegative children aged 4-15 years [9] and in blood from seronegative adults [10]. Furthermore, HCMV pp65 and IE-1 specific $CD4^+$ and $CD8^+$ T-cells were detected in seronegative renal transplant recipients (RTR) [11]. Here we describe seven seronegative adults who presented evidence of HCMV infections. Our investigations illustrate mechanisms which may contribute to their failure to seroconvert. This includes a HCMV-encoded homologue of IL-10 (vIL-10) which interacts with human IL-10R 1 initiating signaling via STAT-3. Its biological activity includes the modulation of cellular IL-10 synthesis. The encoding gene within HCMV (UL111a) generates several splice variants – differentially expressed during latency and lytic infection [12]. HCMV vIL-10 has been detected in plasma [13] with reagents that are available commercially.

## 2. Results

Eighty-two RTR and eighty-one healthy controls were recruited in 2014 and tested for HCMV-reactive IgG in plasma, HCMV UL54 DNA in saliva and vIL-10 in plasma and saliva. There were no differences in gender or ethnicity between the two groups, but the RTR were marginally older ($p=0.07$). Levels of CMV reactive antibodies were determined using in-house ELISAs recognizing a lysate of fibroblasts infected with HCMV AD169 ("HCMV lysate"), gB or IE-1 protein. The cut-off defining seropositivity was 3600 AU/mL based on HCMV lysate [14, 15]. This cut off was determined in relation to samples from individuals deemed to be seronegative using the ARCHITECT CMV IgG assay. Determinations of HCMV serostatus of RTR were concordant with clinical records.

Using this cut-off, 13/82 RTR and 32/81 healthy controls were seronegative ($\chi^2$, $p=0.0007$). Here we present seven individuals who were HCMV seronegative by all three ELISAs. Cases 1, 2 and 3 had detectable HCMV DNA in saliva samples, whilst cases 4, 5, 6 and 7 had detectable HCMV vIL-10 in plasma (Table 1). No HCMV vIL-10 was detected in saliva samples from seronegative or seropositive individuals, but vIL-10 was detectable in plasma from 3/32 seronegative healthy controls and 0/13 seronegative RTR from our cohort in 2014. However one seronegative RTR had detectable vIL-10 in 2012 and 2017. In 2017 4/23 seronegative healthy controls had detectable vIL-10. Three of these also had detectable vIL-10 in 2014.

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**Table 1: Characteristics of seven HCMV seronegative individuals with evidence of HCMV infection**

| | Case 1 | Case 2 | Case 3 | Case 4 | Case 5 | Case 6 | Case 7 |
|---|---|---|---|---|---|---|---|
| Age (years) | 33 | 57 | 62 | 55 | 56 | 34 | 42 |
| Male (M)/Female (F) | F | F | F | M | M | F | F |
| Ethnicity | Caucasian | Caucasian | Caucasian | Caucasian | Caucasian | Asian | Caucasian |
| RTR/ Healthy | RTR | Healthy | Healthy | Healthy | RTR | Healthy | Healthy |
| Donor HCMV status | Negative | - | - | - | Positive | - | - |
| ***T-cell responses (EliSpot assay presented as cells producing interferon-γ/ 200,000 PBMC)*** | | | | | | | |
| HCMV lysate | 0 | 0 | 1 | 0 | 3 | 0 | 3 |
| IE-1 pooled peptides | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| pp65 pooled peptides | 0 | 0 | 1 | 0 | 1 | 0 | 2 |
| ***NK cells (flow cytometry)*** | | | | | | | |
| FcRγ- (% $CD3^{-}CD56^{dim}$) | 8.5% | 27.3% | 2.7% | 3.9% | 8.8% | 4.1% | 13.8% |
| $NKG2C^{+}$ (% $CD3^{-}CD56^{dim}$) | 2.3% | 7.1% | 4.3% | 1.8% | 2.1% | 2.8% | 2.8% |
| ***γδ T-cells (flow cytometry)*** | | | | | | | |
| Vδ2- (% CD3+) | 0.19% | 0.5% | 2.2% | 0.1% | 0.4% | 0.7% | 1.2% |
| ***HCMV DNA / miRNA*** | | | | | | | |
| HCMV DNA in saliva (UL54 qPCR) | Pos | Neg | Pos | Neg | Neg | Neg | Neg |
| HCMV DNA in saliva (UL55 nested PCR) | Pos | Pos | Pos | Neg | Neg | Neg | Neg |
| HCMV-encoded miRNA in blood | Neg | Pos (miR-US5-2-3p) | Neg | NT | NT | Neg | NT |
| ***Plasma CMV vIL-10 (pg/mL)*** | | | | | | | |
| 2006 | NA | NA | NA | NA | NA | 801 | NA |
| 2012 | <60 | NA | NA | 1840 | 945 | NA | NA |
| 2014 | <60 | <60 | <60 | 639 | <60 | 1440 | 166 |
| 2017 | NA | <60 | <60 | 916 | 704 | 1247 | 786 |
| 2021 | NA | NA | NA | NA | NA | 815 | NA |

NT not tested; NA no sample available

*Three individuals were seronegative despite deteactable HCMV DNA*

Case 1 is a 33-year-old female RTR who received a donor kidney six years before sample collection She was free of graft rejection, diabetes, HCMV or cardiac disease since transplantation and received stable doses of immunosuppression (tacrolimus, mycophenolate mofetil and prednisolone). The transplanted kidney was from a HCMV-seronegative donor. Accordingly, there was no detectable HCMV IgG, IgA and IgM in saliva or plasma (Table 1 and unpublished data). Case 1 was HCMV DNA positive in saliva by our in-house qPCR assays detecting MIE and UL54, but negative in plasma and buffy coat. Sanger sequencing of amplicons encompassing gB produced by nested PCRs of saliva identified a mixed infection where the predominate genotype was gB2. The presence of multiple strains was confirmed by sequencing genes encoding the NK cell receptor homologues UL18 and UL40 (data not shown). HCMV vIL-10 was not detectable in plasma in 2014 or 2017.

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Case 2 is a 57-year-old female healthy control who was seronegative for HCMV IgG in plasma and HCMV DNA negative by qPCR assays targeting MIE and UL54 in plasma and the associated buffy coat samples. However she had detectable HCMV DNA in saliva when assessed with the nested PCR targetting gB (UL55). Sanger sequencing identified a mixed infection where the predominate genotype was gB2. HCMV-miR-US5-2-3p was also detected in her saliva [16]. Neither IgA, IgG and IgM antibodies reactive with HCMV antigens nor HCMV vIL-10 were detectable in saliva or plasma (Table 1; data not shown). Analysis of her PBMC by flow cytometry revealed expanded populations of FcγR$^-$ and NKG2C$^+$ NK-cells, characteristic of HCMV infection [17, 18] (Table 1). The FcγR$^-$ population comprised 27.3% of CD3$^-$CD56$^{dim}$ NK-cells, compared with a median (range) of 9.1 (5.4-19.1)% for other seronegative healthy controls (Figure 1). The NKG2C$^+$ population comprised 7.1% of CD3$^-$CD56$^{dim}$ NK-cells, compared with a median (range) of 2.6 (0.9-4.0)% for other seronegative healthy controls (Figure 1). The same phenotypes were assessed at 2017 showing populations with 15.5% FcγR$^-$ and 11.1% NKG2C$^+$ CD3$^-$CD56$^{dim}$ cells. In 2017, she had not seroconverted and no HCMV DNA was detected in saliva. This case establishes the possibility that NK cells may control HCMV replication without seroconversion.

Case 3 was also a healthy female with detectable HCMV DNA in her saliva. She was seronegative in 2014 and 2017 , and had no T-cell responses when assessed against three HCMV antigens (Table 1). HCMV vIL-10 was not detectable in plasma in 2014 or 2017, and no expanded populations of FcγR$^-$ and NKG2C$^+$ NK-cells were evident. Expanded populations of Vδ2- γδ T-cells are associated with HCMV seropositivity in RTR and healthy controls [19]. Case 3 had an expanded population of Vδ2$^-$ γδ T-cells (2.2% of all γδ T-cells; Table 1), compared with a median (range) of 0.47% (0.06-2.08%) for other seronegative healthy controls and 0.96% (0.05-3.8%) for seropositive healthy controls.

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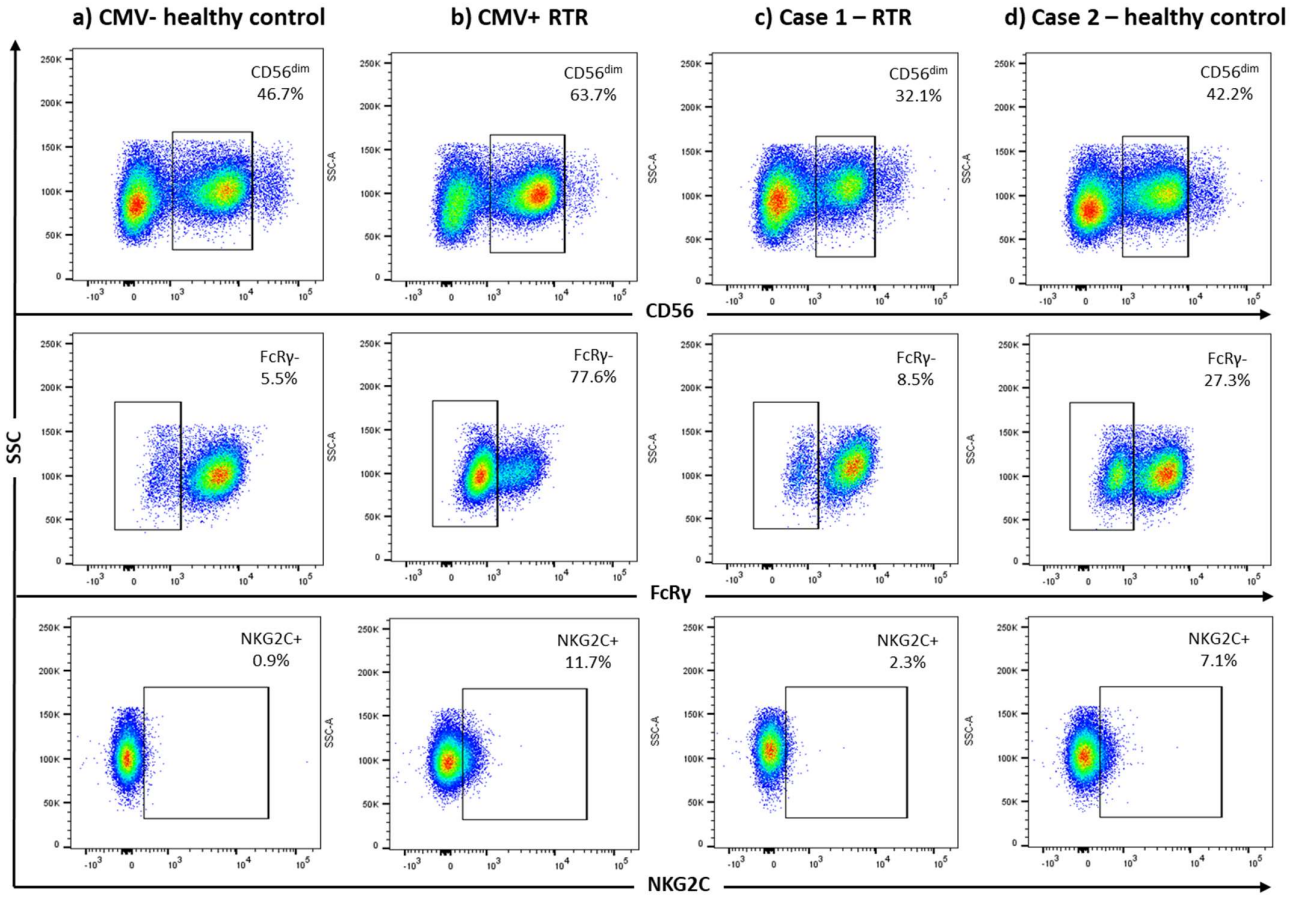


**Figure 1. FcRγ and NKG2C expression on NK cells.** Representative flow cytometry plots for a CMV seronegative healthy control (a) and a CMV seropositive renal transplant recipient (RTR) (b). Flow cytometry plots for Case 1 (c) and Case 2 (d). For the gating strategy, singlets were first defined by forward scatter area (FSC-A) and forward scatter height, lymphocytes were then gated based on side scatter area and FSC-A, and dead cells were excluded based on uptake of Fixable Viability Stain. Lack of FcRγ expression (middle panel) and expression of NKG2C (bottom panel) were assessed in NK cells identified as $CD3^{-}CD56^{dim}$ (top panel).

*Four seronegative inidivduals had detectable HCMV vIL-10 in plasma*

Case 4 is a 55-year-old male healthy control who was seronegative for HCMV IgG in plasma by our in-house ELISA assays and HCMV DNA negative by our in-house qPCR assay targeting UL54 in saliva. He works in a hospital environment and is frequently exposed to patients with active HCMV infections, but remained seronegative when tested in 2012, 2014 and 2017. However HCMV vIL-10 was detected in his plasma at all three time points [1840, 639 and 916 pg/ml resp *vs* median(range): 166 (0-1440) for seronegative healthy controls when tested in 2014. The case establishes the possibility that vIL-10 may suppress seroconversion. This is supported by Cases 5, 6 and 7 (Table 1). Case 5 was an RTR who failed to seroconvert when given a HCMV-positive donor kidney. Case 6 remained seronegative with detectable plasma vIL-10 from 2006 to 2021.

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## 3. Discussion

HCMV seronegativity is widely assumed to define an individual who is not carrying the virus and has not done so recently. However from a cohort of 32 individuals we have identified seven cases who remained HCMV seronegative despite evidence of current HCMV replication or latent carriage – specifically HCMV DNA, miRNA, vIL-10, and/or FcγR$^-$ NK cells or Vδ2$^-$ γδ T-cells characteristic of HCMV seropositivity. Cases 1, 2 and 3 had HCMV DNA in saliva, and Case 2 also had detectable HCMV-miR-US5-2-3p [16]. Case 2 had a striking population of FcRγ$^-$ and NKG2C$^+$ NK cells in circulation. Several studies have linked this population with HCMV seropositivity [17]. We have linked detection of UL54 HCMV DNA in saliva from the same cohort of RTR with systemic markers of HCMV infection [5]. Here we show that Case 2 cleared the salivary infection without seroconversion, since she was DNA and antibody negative in 2017. Her NK cell response may have favoured compartmentalization to the saliva. This may also be achieved through intrinsic immunity mediated by restriction factors (RS) such as interferon gamma-inducible protein 16 (IFI16) which can prevent HCMV DNA sensing by inhibiting UL54 (DNA polymerase) by interacting with CMV pp65. [20]. IFI16 has been found in saliva [21]. Case 3 had an expanded populations of Vδ2- γδ T-cells which may have cleared the virus preventing seroconversion [22], despite the CMV DNA found in her saliva.

Cases 4-7 had measurable levels of HCMV-encoded vIL-10 in their plasma which were relatively stable over several years. No individuals had no detectable HCMV-reactive T-cells in 2014. Case 6 had detectable vIL-10 over a 15-year period (2006-2021). All samples were tested for HCMV-reactive antibodies and remained negative. Case 6 is known to be seropositive for Epstein-Barr virus (EBV) suggesting that the suppression of a humoral response is HCMV-specific. The detection of vIL-10 may reflect transcription of the encoding gene (UL111a) during HCMV latency [23], explaining the lack of detectable HCMV DNA.

We considered the possibility that the presence or absence of detectable vIL-10 was determined by variations in UL111a [20]. Viruses identified in cases 1, 2 and 3 were subjected to amplicon-based enrichment for next generation sequencing of a selection of HCMV genes, including UL111a. The sequence from all three cases had variations when compared to the Toledo reference strain but none were unique to these three samples – ie: they were also seen in viruses sequenced from HCMV seropositive HIV$^+$ individuals from Jakarta (Indonesia) and RTR and/or healthy controls from Perth, Australia (manuscript in preparation).

The detection of vIL-10 was common in the seronegative samples tested (4/32 healthy adults and 1/13 RTR tested between 2014-2017). A simple explanation would be that the vIL-10 blocks seroconversion, but proof of this is lacking. The individuals described here have no clinically recognized immune defect, so any suppressive effects of vIL-10 must be specific to HCMV itself. The individuals also had no T-cell responses to three HCMV antigens (Table 1), which may suggest the vIL-10 is blocking T-cell responses as well. The biological effects of vIL-10 include enhanced signaling of the CXCR4/CXCL12 pathway triggered by the chemokine receptor homologue US27 [24]. This is distinct from the Stat3-dependent effects of vIL-10 which include modulation of dendritic cell function and macrophage activation [25].

Blockage of HCMV seroconversion by vIL-10 has implications for the efficacy of HCMV vaccines. HCMV vaccines currently in phase I or II clinical trials seek humoral responses to selected HCMV antigens, but their efficacy remains low (i.e. <50% [26]). Indeed a blockade of vIL-10 has been proposed as a vaccine strategy [25]. Our data supports further development of this approach.

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## 4. Conclusions

We have shown that a proportion of HCMV seronegative adults present evidence of active or latent HCMV infections. These may be transient with control by NK cells (as in case 2) or persistent with seroconversion blocked by vIL-10. It is clear that seropositivity is a risk factor for adverse outcomes following renal transplantation [27]. However some seronegative individuals still face complications following organ transplantation and HCMV may play a role.

## 5. Materials and Methods

*Study Cohort*

Eighty-two RTR were recruited from renal clinics at Royal Perth Hospital (Western Australia). Inclusion criteria were clinical stability >2 years after transplant, no CMV disease or reactivation within 6 months of sample collection and no current anti-viral treatment. RTR with hepatitis B or C were excluded. Healthy adults recruited as controls were matched with the RTR by age and gender [14]. Ethics approval was obtained from Royal Perth Hospital Human Research Ethics Committee (EC 2012/155) and endorsed by Curtin University Human Research Ethics Committee (HR16/2015; HRE2021-0044). Participants provided written informed consent. Here we present case studies of five healthy controls and two RTR.

*Immunological responses to HCMV*

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density centrifugation. Plasma was stored in -80°C and PBMC in liquid nitrogen. Plasma HCMV IgG titers were assessed using in-house ELISAs based on a lysate of fibroblasts infected with HCMV AD169, recombinant CMV gB (Chiron Diagnostics, Medfield, MA) or IE-1 protein (Miltenyi Biotech, Cologne, Germany). Results were presented as arbitrary units (AU)/mL based on a standard plasma pool [13,14]. $FcR\gamma^-$ and $NKG2C^+$ NK cells ($CD3^-CD56^{dim}$) and Vδ2- γδ T-cells were enumerated using multicolor flow cytometry, as the populations are expanded in CMV-seropositive RTR [18, 19]. Enzyme linked immunosorbent spot (ELISpot) assays utilized anti-IFNγ antibodies (MabTech, Stockholm, Sweden). Cells were stimulated with CMV lysate, CMV pp65 peptide pool (NIH AIDS reagent program, Woburn, MA) or a CMV IE-1 peptide pool (JPT, Berlin, Germany). Numbers of spots in unstimulated wells were subtracted from numbers in stimulated wells and adjusted per 2 x $10^5$ PBMC.

*vIL-10 ELISA*

Levels of HCMV vIL-10 were assessed using a sandwich ELISA. 96-halfwell plates were coated overnight at 4ºC with 50µL/well anti-CMV vIL-10 goat polyclonal antibody (AF117) at 2µg/mL. The plates were washed three times with 150µL/well of PBS-0.005% TWEEN and blocked for 1 hour with 1% BSA/PBS. A standard curve from 4000pg/mL to 62.5 pg/mL was created using recombinant HCMV vIL-10 with carrier protein (117-VL-025). Plasma samples were diluted 1:10 in 1%BSA/PBS and a QC was created by spiking a plasma sample with 500pg/mL of standard (CV=17%). 50uL/ well of anti-CMV vIL-10 goat polyclonal biotinylated antibody (BAF117) at 0.2µg/mL was added (2 hours, room temperature), followed by streptavidin-HRP conjugate (30 minutes, room temperature) and TMB substrate (20 minutes, room temperature). Reactions were stopped with 25uL/well 1M $H_2SO_4$ and read at 450nm. Concentrations of vIL-10 were interpolated from the standard curve [28]. The minimum level detected by this assay was 62.5 pg/mL.

 doi:10.20944/preprints202107.0565.v1



*Detection of CMV DNA in Saliva*

Saliva (approximately 5 mL) was collected after a water mouth wash by asking the participant to spit into a tube. Samples were centrifuged (1000g, 10 min). DNA was extracted from the pellet using FavorPrep Blood Genomic DNA Extraction Mini Kits (Favorgen, Ping-Tung, Taiwan) and stored at -80°C. Each DNA extraction run included a no-sample control with saliva replaced by PBS. HCMV was detected using an in-house qPCR assay with primers targeting the UL54 gene (CMV DNA polymerase) presented in Table 2 [5]. Quantitation was achieved using a standard curve created using DNA extracted from a lysate of HCMV (AD169)-infected fibroblasts and serially diluted 10-fold. Samples were considered positive if a steady amplification curve was initiated before 38 cycles (the lowest point on the standard curve). Positive results were normalized against the gene encoding beta-2-microgobulin and values were reported in arbitrary units (range: 44–721) [29]. Some samples also underwent amplification of MIE by qPCR using the same protocol as UL54 with the primers described in Table 2. All qPCR runs included at least two no-template controls, with PBS controls from the DNA extractions spread across a series of qPCR runs. No amplicons were detected.

To achieve greater sensitivity, samples were also assessed with a nested PCR to detect UL55 [30] (gB; see Table 2), using PCR buffer with 35 mM MgCl2 for the inner reaction and 30 mM $MgCl_2$ for the outer PCR in a total volume of 20 µL, with 1 µL of each primer (10 µM), 2 µL PCR buffer, 1 µL 40 mM dNTPs, 0.2 uL Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA), 5 µL DNA (diluted 1:2) for the outer PCRs and 3µL the outer PCR product for the inner PCRs. The cycling conditions for outer PCRs were: 1 cycle of 5 minutes at 95°C followed by 35 cycles of 30 seconds at 95°C and 30 seconds at 60°C and then 1 cycle of 1 minute at 72°C. The inner PCRs ran only 30 cycles. PCR products from the inner PCRs were run on 1% agarose gel in 0.5× TBE buffer stained with GelGreen (Biotium, Fremont, CA) for 1 hour at 120 volts. Amplicons were sent for purification and Sanger sequencing by the Australia Genome Research Facility (AGRF). All nested PCR runs included a no-template control carried from the outer PCR to the inner PCR, a no-template control for the inner PCR and DNA extracted from uninfected fibroblasts. No amplification was detected.

*Detection of CMV-encoded miRNA in Saliva*

Saliva pellets were thawed and mixed with TRI reagent (1:4) before RNA extraction using the MagMAX—96 for Microarrays kit as described previously [16] (Applied Biosystems, Foster City, CA, USA). Custom reverse transcription primer pools were generated, and cDNA synthesis for all miRNA assays was performed in a single reaction, according to the manufacturer's protocols (Applied Biosystems, PN 4465407). A pre-designed primer and probe assay targeting mature miRUS5-2-3p (assay ID: 469255_mat was purchased from Applied Biosystems). RNA from HCMV seronegative healthy participants and uninfected THP-1 cells were used to ensure specificity. These showed no amplification up to 40 cycles. Sensitivity was determined using 10-fold serial dilutions of HCMV AD169 RNA. Samples with cycle thresholds below $10^4$ dilution of the standard (i.e., after cycle 32–36, depending on the miRNA assayed) were considered negative. All samples were run two to four times and called positive if at least two replicates produced amplification.

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**Table 2. Primer and probe sequences used to detect HCMV DNA by qPCR or nested PCR**

| Target | Primer | Sequence (5′-3′) | Product (base pairs) |
|---|---|---|---|
| UL54 | FWD | CCCGAAAACGTGTCGCC | 105 |
| | REV | AAACGTTGACGCAGATACTGTAGC | |
| | PROBE (5 μM) | 6-FAM-TATCGTCAGCATCTGGTGC -BHQ-1 | |
| MIE | FWD | AACTCAGCCTTCCCTAAGACCA | 76 |
| | REV | GGGAGCACTGAGGCAAGTTC | |
| | PROBE (2 μM) | 6-FAM-CAATGGCTGCAGTCAGGCCATGG-TAMRA-6 | |
| B2M | FWD | TGAGTATGCCTGCCGTGTGA | 105 |
| | REV | ACTCATACACAACTTTCAGCAGCTTAC | |
| | PROBE (5 μM) | 6-FAM-CCATGTGACTTTGTCACAGCCCAAGATAGTT- TAMRA-6 | |
| gB (UL55) | OUTER FWD | GAATRGCTGAYGGRTTGATCTTG | 590 |
| | OUTER REV | GATCTCCTGGGATATACAGGACG | |
| | INNER FWD | GAGTTCCTTGAAGACCTCTAG | 519 |
| | INNER FWD | ACYTTCTGGGAAGCCTCGGAACG | |

**Author Contributions:** For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, S.W. and P.P; methodology, S.W., S.L. and P.P; formal analysis S.W.; investigation, S.W. and P.P.; resources, P.P and A.I.; data curation, S.W. and S.L.; writing—original draft preparation, S.W.; writing—review and editing, P.P., S.L., and A.I.; visualization, S.W.; supervision, P.P. and S.L.; project administration, P.P.; funding acquisition, P.P. All authors have read and agreed to the published version of the manuscript."

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**Conflicts of Interest:** The authors declare no conflict of interest.

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