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ORIGINAL ARTICLE

Cytomegalovirus infections in HIV/AIDS patients: prevalence, disease-associated factors and ganciclovir resistance

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Abstract

Introduction: Cytomegalovirus (CMV) is an opportunistic pathogen that causes various end-organ diseases. In HIV patients, it is linked to unfavourable progression even in the era of anti-retroviral therapy (ART). Cases of CMV drug resistance may worsen the situation. This study aimed to determine the prevalence of CMV-HIV co-infection, its associated factors and genotypic detection of ganciclovir-resistant CMV. *Materials and Methods:* The clinical and laboratory data of 358 HIV patients clinically suspected of CMV infections from December 2018 to December 2020 in Sungai Buloh Hospital, Selangor, Malaysia, were retrospectively analysed. Forty samples were tested for ganciclovir-resistant UL97 mutations (M460I and M460V) using a high-resolution melting curve (HRM) and Sanger sequence analysis. *Results:* The prevalence of CMV infection among HIV patients, detected in plasma, cerebrospinal fluids, vitreous fluids and tissue specimens by CMV PCR, was 60.3% (216/358). Pneumonitis (100/216, 46.3%) and gastrointestinal diseases (65/216, 30.1%) were the predominant clinical presentations of CMV-HIV co-infection, followed by retinitis 5.6% (12/216). The majority of HIV patients (84.6%) who succumbed to death were co-infected with CMV. There were significant associations ($p<0.05$) between ART status, HIV viral load and CD4 cell count with CMV infection when tested individually. In multivariate analysis, CD4 cell count showed significant association, where decreased CD4 cell count increases the likelihood of CMV infection. No ganciclovir-resistant mutations were detected. *Conclusion:* Despite the high prevalence of CMV infection, the absence of ganciclovir-resistant strain is a good indication. However, the possibility of drug resistance by other gene mutations in different codons cannot be ruled out using this HRM method.

Keywords: cytomegalovirus infection, HIV, drug resistance, ganciclovir, sequence analysis.

INTRODUCTION

Cytomegalovirus (CMV) is a double-stranded DNA virus, which belongs to the family *Herpesviridae,* subfamily *Betaherpesvirinae,* genus Cytomegalovirus*,* and species *Human betaherpesvirus 5*. It is a ubiquitous virus, reported worldwide with more than 80% seroprevalence in the general population.[1,2] It persists as an important opportunistic viral infection in HIV/AIDS, although the morbidity and mortality have improved with the introduction of highly active antiretroviral therapy.[3] Various clinical presentations of CMV infection were described globally, with retinitis reported as the most typical presentation in the former decades.[4] Meanwhile, systemic illness and respiratory infections were increasingly reported in a recent study.[5] CMV also significantly

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affects HIV acquisition, HIV disease progression, morbidity, and mortality.[6,7] These were attributed to the utilisation of alternative CMV co-receptor pathways that enhance HIV viral uptake and cell tropism, reactivation of latent HIV in T-cells, and augmentation of HIV replication in CMV co-infected cells.[6,8] In the era of anti-retroviral therapy (ART), a low CD4 cell count, CMV viremia, and pp65 antigenemia remain as risk factors for CMV disease development.[9,10] A low CD4 count and a high level of HIV viral load were also found to be significant factors for CMV infection in the HIV population.[11]

The first-line treatment for CMV diseases is ganciclovir/valganciclovir, with a duration of 14-21 days, followed by continuation of the antiviral maintenance therapy in patients with low immune recovery.[2] Foscarnet, mirabavir and CXM001 are alternative antivirals for treating drug-resistant CMV.[12] The major setback of ganciclovir is its myelotoxicity, which resulted in leukopenia, anaemia and thrombocytopenia, and is ineffectively monitored by therapeutic drug monitoring (TDM).[13] A prolonged antiviral exposure, insufficient concentration of the drug at the site of infection, intermittent drug interruption, and profound immunocompromise will contribute to the development of the drug-resistant CMV strains.[12] Nevertheless, high mortality was found in HIV patients with drug-resistant CMV diseases, hence raising the importance of effective laboratory testing to detect the drug-resistant strains.[4] Phenotypic detection of drug-resistant CMV is carried out by observing the inability of the virus to be inhibited by the specified concentration of a specific antiviral. Genotypic testing, on the other hand, is achieved by detecting the presence of mutations in the UL97 protein that confers resistance to ganciclovir, or in the UL54 protein that results in resistance to ganciclovir, cidofovir, and foscarnet.[14–16] Mutations in UL97 protein were found in approximately 90-95% of ganciclovir-resistant CMV strains.[17,18] In Malaysia, information on CMV and HIV co-infection, genotypic characterisation of the locally circulating CMV virus, and the emergence of drug-resistant strains were limited. Here, we studied the prevalence of CMV and HIV co-infection, its association factors, and genotypic testing of ganciclovir-resistant CMV among the HIV population.

MATERIALS AND METHODS

Study population

A cross-sectional study was conducted from December 2018 to December 2020 in Sungai Buloh Hospital, Malaysia, a national referral centre for Infectious Diseases. The individuals included in the study were those previously diagnosed with HIV and who were clinically suspected of CMV diseases, ranging in age from 18 to 69 years old. Individuals with a history of organ transplantation or immunocompromised conditions other than HIV/AIDS were excluded. Clinical specimens consisting of plasma, cerebrospinal fluid (CSF), tissues (e.g. colon biopsy) and vitreous fluid from these patients were sent to the microbiology laboratory for diagnostic testing as part of the standard of care.

Ethical approval for this study was obtained from Medical Research and Ethics Committee (MREC), Ministry of Health, Malaysia (NMRR-19-3961-51825), and Research Ethics Committee, Universiti Kebangsaan Malaysia (JEP-2020-148).

CMV case definition

It is important to correctly define CMV cases to be used in both clinical and research settings. This is because CMV infection and CMV end-organ diseases do not inevitably occur concurrently in HIV patients. Moreover, definitive diagnosis of CMV end-organ disease is seldom achieved due to the invasive nature of histopathology sampling. Adapting the definition described by a well-established consensus in the solid organ transplant community, "CMV infection" refers to virus isolation or detection of viral proteins (antigens) or nucleic acid in any body fluid or tissue specimen regardless of symptoms or signs, whereas "CMV disease" refers to evidence of CMV infection with attributable symptoms or signs and CMV disease may manifest as either a viral syndrome (e.g., fever, malaise, leukopenia, neutropenia, atypical lymphocytosis, thrombocytopenia) or as a tissue-invasive disease.[19,20] In this study, the "CMV-infected" group will be referring to the patients with CMV DNA PCR detected in the clinical specimens, while "CMV non-infected" indicates the group with negative CMV DNA PCR.

CMV DNA quantitation and sample selection

Diagnostic quantitative CMV DNA was carried out using CMV PCR kit (GeneProof® CMV PCR kit, Bruno, Czech Republic) in Rotor-Gene

Q Real-time PCR instrument (Qiagen, Hilden, Germany). All the samples were stored in -70 °C freezer after the initial CMV DNA PCR. The archived samples with CMV viral copies of more than 1,000 IU/ml, with a sufficient volume of >400µl, and stored for less than a year were selected and processed for genotypic testing of UL97 mutation. Only samples with a CMV viral load of more than 1,000 IU/ml were processed for further testing in this study. This is due to the limitation of genotypic testing and the correlated clinical significance of infection. A previous study found that CMV viral loads of fewer than 1,000 copies/ml are often associated with failure of genomic analysis by Sanger sequencing.[21] In addition, interpreting a CMV viral load of less than 500 copies/ml is challenging and may not accurately represent clinical circumstances or be related to end-organ diseases.[22]

Sample preparation / Nucleic acid extraction
DNA was extracted from the clinical specimens using croBEE® 201A Nucleic Acid Extraction Kit. A 10 µl Carrier RNA (1 mg/ml) and 20 µl Proteinase K (10 mg/ml) were added to 400 µl clinical specimens. The prepared specimens and the pre-filled Cartridge Reagent were then inserted into the croBEE® NA16 Nucleic Acid Extraction System. The 201 programs proceeded to get an elution product of 60 µl viral DNA, and the extracted DNAs were ready for genotypic testing of UL97 protein mutation.

Genotypic testing of UL97 protein mutation
The genotypic testing of the UL97 mutation for CMV is performed using a high-resolution melting (HRM) PCR assay. HRM was designated in previous studies as a suitable test for the detection of M460I and M460V mutations of UL97 protein, which confer resistance to ganciclovir.[23,24] The primers were verified using online software (PrimerQuest™ Tool IDT), and the sequences are listed in TABLE 1. The PCRs were performed using Type-IT HRM PCR Qiagen Kit (Qiagen, Courtabeuf, France) on a Rotor-Gene Q Real-time PCR instrument (Qiagen, Hilden, Germany). The following program was used for cycling: initial denaturation at 95°C for 5 min; denaturation at 95 °C for 10 s; annealing at 60 °C for 30 s; extension Optics ON at 72 °C for 10 s, repeat for 45 cycles; heteroduplex formation at 95 °C for 30 s and at 50 °C for 30 s; followed with melting curve analysis with HRM start at 80 °C to 95 °C with rate of 0.1 °C/s. The product size of UL97 PCR is 470 bps. The wild-type strains of UL97 had a melting point (Tm) at 89.49 °C, while the point mutation in codon 460 increased the melting point (Tm) to 89.71 °C. The wild-type control was generated based on a partial sequence of the human CMV AD169 (UL97) gene obtained from GenBank (Accession no: KX772749.1). Similarly, the mutant control was generated based on the fragment sequence of the human CMV M460V mutant (UL97) gene, obtained from GenBank (Accession no: U07356.1). The sequences of the controls are stated in TABLE 1. Sanger sequencing is performed directly using the PCR product on the selected samples for confirmation. The sequences were analysed for M460V or M460I mutation using a bioinformatics website; CMV mutation resistant analyser Universität Ulm, AG Bioinformatics and Systems Biology, Institute of Neural Information Processing, Institute of Virology. All the sequences were also assessed for nucleic acid similarity using the Basic Local Alignment Search Tool (BLAST), NCBI website. The flowchart of the study is illustrated in FIG 1.

Statistical Analyses
The data analysis was done using the SPSS version 22. Descriptive data is expressed as mean ± standard deviation (SD) unless otherwise stated. Categorical data was analysed using Chi-square or Fisher's exact test. Multiple logistic regressions were used for multivariate analysis of the association factors. The result of regression analysis was expressed as an adjusted odds ratio with a 95% confidence interval. A value of p-value <0.05 is considered statistically significant.

RESULTS

Prevalence and demographic characteristics of CMV co-infected HIV patients
Out of 358 HIV patients clinically suspected of CMV disease, 216 (60.3%) had CMV DNA detected in the clinical specimens ("CMV infection"). The CMV-positive samples comprised of 201 (93.0%) plasma, 9 (4.2%) CSF, 3 (1.4%) vitreous fluid, and 3 (1.4%) tissue specimens. The CMV co-infected HIV patients were predominantly male, ranging from 21 to 69 years old, with a mean age of 38. Pneumonitis and gastrointestinal diseases were the predominant clinical presentations of HIV with "CMV disease", with a percentage of 46.3% (100/216) and 30.1% (65/216), respectively, while retinitis was 5.6% (12/216) of the total

TABLE 1: Primer and control sequences used in high-resolution melting (HRM) PCR for the detection of UL97 mutation

Sequence label	Sequence, 5' to 3'
Primer	
UL97F (141693– 141712) Forward	CTGCTGCACAACGTCAAGGT
UL97R (142131–142153) Reverse	CCCAGCGCCGACAGCTCCGACAT
Controls	
UL97 Wild Type (AD169)	GCCACGGGCTGCTGTCTGCTGCACAACGTC AAGGTACATCGACGTTTCCACACAGACATG TTTCATCACGACCAGTGGAAGCTGGCGTGC ATCGACAGCTACCGACGTGCCTTTTGCACG TTGGCCGACGCTATCAAATTTCTCAATCACC AGTGTCGTGTATGCCACTTTGACATTACACC CATGAACGTGCTCATCGACGTGAACCCGCA CAACCCCAGCGAGATCGTGCGCGCCGCGCT GTGCGATTACAGCCTCAGCGAGCCCTATCC GGATTACAACGAGCGCTGTGTGGCCGTCTT TCAGGAGACGGGCACGGCGCGCCGCATCCC CAACTGCTCGCACCGTCTGCGCGAATGTTA CCACCCTGCTTTCCGACCCATGCCGCTGCAG AAGCTGCTCATCTGCGACCCGCACGCGCGT TTCCCCGTAGCCGGTCTACGGCGTTATTGCA TGTCGGAGCTGTCGGCGCTGGGCAACGTGC TGGGCTT
UL97 Mutant (M460V)	GCCACGGGCTGCTGTCTGCTGCACAACGTC ACGGTACATCGACGTTTTCACACAGACATG TTTCATCACGACCAGTGGAAGCTGGCGTGC ATCGACAGCTACCGACGTGCCTTTTGCACG TTGGCCGACGCTATCAAATTTCTCAATCACA AGTGTCGTGTATGCCACTTTGATATTACACC CGTGAACGTGCTCATCGACGTGAACCCGCA CAACCCCAGCGAGATCGTGCGCGCCGCGCT GTGCGATTACAGCCTCAGCGAGCCCTATCC GGATTACAACGAGCGCTGTGTGGCCGTCTT TCAGGAGACGGGCACGGCGCGCCGCATCCC CAACTGCTCGCACCGTCTGCGCGAATGTTA CCACCCTGCTTTCCGACCCA TGCCGCTGCAG AAGCTGCTCA TCTGCGACCCGCACGCGCGT TTCCCCGTAGCCGGCCTACGGCGTTATTGCA TGTCGGAGTTGTCGGCGCTGGGTAACGTGC TGGGCTT

The underlined nucleotides shows corresponding sequences of codon 460 in UL97 Wild Type strain (ATG) and in UL97 M460V mutation strain (GTG)

cases. The overall prevalence of CMV retinitis among all HIV patients was only 3.3% (12/358). Furthermore 69.4% (150/216) of the CMV co-infected HIV patients had other concomitant or opportunistic infections (OIs) during the same presentation. *Mycobacterium tuberculosis* (MTB) infections and *Pneumocystis jirovecii pneumonia* (PJP) were the two most prevalent concurrent opportunistic infections, comprising 37% (55/150) and 26% (39/150) of the cases, respectively. The remaining concurrent infections included penicillosis, invasive candidiasis, bacterial infections, syphilis, and cerebral toxoplasmosis.

The majority of the CMV co-infected HIV patients (78.2%) were either ART-naïve or ART defaulters for more than three months, corresponding to 43.5% (94/216) and 34.7%

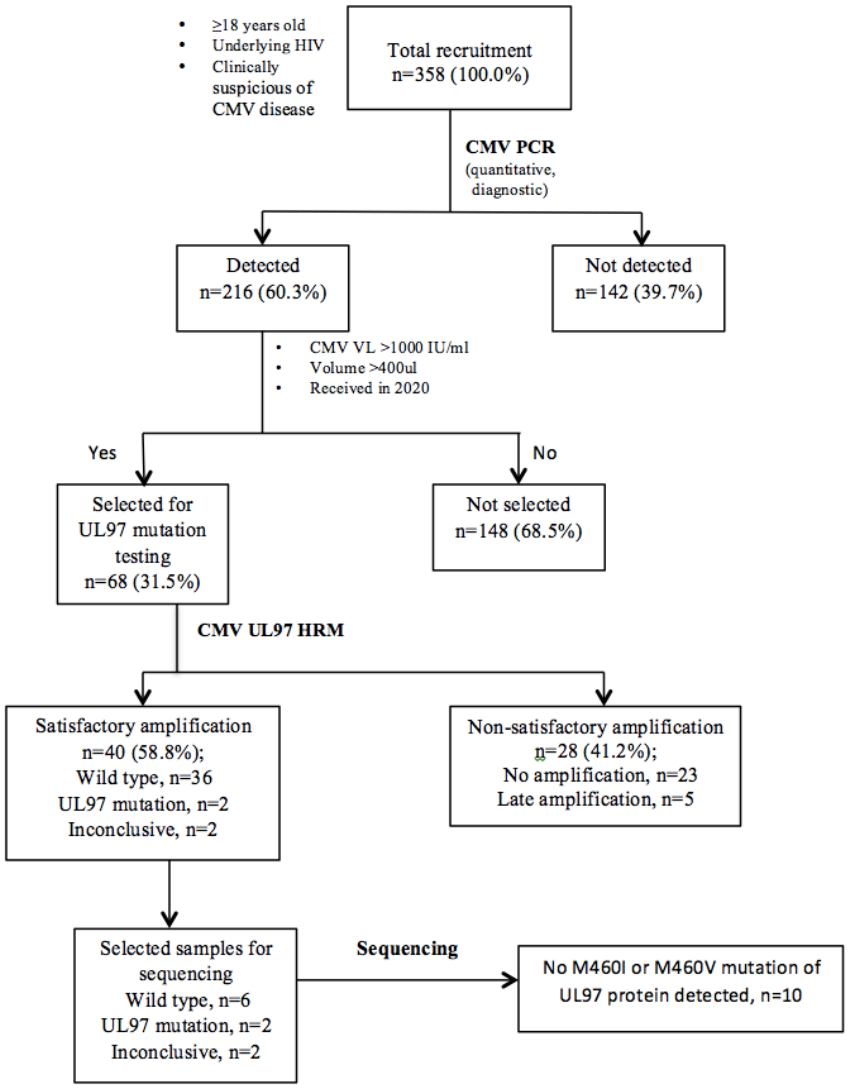

FIG. 1 Study flowchart

(75/216), respectively. Only 14.8% (32/216) of the patients were on regular ART for over a month, and about 6.9% (15/216) had just started ART within a month of the current presentation. In terms of CMV treatment, 17.6% (38/216) of the CMV co-infected HIV patients received intravenous ganciclovir for 2 weeks duration, 18.5% (40/216) were treated for more than 2 weeks, and 9.7% (21/216) had an incomplete course of fewer than 2 weeks due to various reasons including intolerability and haematological toxicity. The other 54.2% (117/216) had no documented ganciclovir therapy.

The mean CMV viral copies were 5,046,393 IU/ml, while the median was 174,685 IU/ml and was not normally distributed. The mean HIV viral load of the CMV co-infected HIV patients was 618,627 copies/ml, with a median of 174,685 copies/ml. The HIV viral load was not normally distributed, and the highest proportion (52.8%) of the patients had an HIV viral load of more than 5 log10 copies/ml. Similarly, the CD4 cell count of all the patients was not normally distributed, with a mean of 50 cells/µl; the majority (94.0%) had CD4 cells of less than 200 cells/µl. The rest of the demographic distributions are tabulated in TABLE 2.

CMV DNA detection rate

Analysis of the CMV DNA PCR across different specimen types showed a higher detection rate in plasma samples (69.3%) as compared to CSF (15.3%) and tissue specimens (50%) (TABLE 3).

TABLE 2: Demographic characteristics of the CMV co-infected HIV patients

Characteristics	n=216	n	%
Gender	Male	197	91.2
	Female	19	8.8
Age (years)	18-30	53	24.5
	31-50	134	62.0
	51-70	29	13.4
Ethnicity	Malay	146	67.6
	Chinese	45	20.8
	Indian	17	7.9
	Others	8	3.7
Clinical presentation of "CMV disease"	Pneumonitis	100	46.3
	GIT diseases[a]	65	30.1
	Encephalitis	26	12.0
	Retinitis	12	5.6
	Disseminated disease	6	2.8
	Bone marrow suppression	4	1.9
	HPB diseases[b]	3	1.4
ART status	ART naïve	94	43.5
	Default ART >3 months	75	34.7
	ART <1month	15	6.9
	On ART >= 1 month	32	14.8
HIV viral load (log10)	0-1.3	9	4.0
	1.4-3.0	41	19.0
	3.1-5.0	52	24.0
	>5.0	114	53.0
CD4 cell count (cell/µl)	0-100	181	83.8
	101-200	22	10.2
	>200	13	6.0
Other opportunistic infection	Yes	150	69.4
	No	62	28.7
	Not documented	4	1.9
30-days outcome	Alive	183	84.7
	Died	33	15.3

[a]GIT (gastrointestinal) diseases: oesophagitis, gastroenteritis, colitis. [b]HPB (hepatobiliary) diseases: pancreatitis, hepatitis and cholangitis.

The 100% detection rate of vitreous fluid is most likely contributed by prior ophthalmology assessment of CMV features that guide the sampling decision towards those with positive clinical findings while leaving out those with negative ophthalmological findings. The CMV DNA detection rate also differs with different clinical presentations, where patients who presented with pneumonitis, gastrointestinal symptoms, and hepatobiliary and disseminated diseases had a higher detection rate of more than 70%. In comparison, patients with encephalitis showed a lower CMV detection rate of less than 30% in the clinical specimens (TABLE 3). However, this finding did not take into account the discordance between the symptoms and specimen; for example, certain patients presented with encephalitis only had plasma specimens

TABLE 3: Univariate analysis on the association between CMV infection with clinical background, antiretroviral therapy (ART) status, HIV biomarkers, and mortality (n=358)

Variables	CMV infected		CMV non-infected		x^2	df	*p*-value
	n	%	n	%			
Overall	216	60.3	142	39.7			
Gender					0.955	1	0.371
Male	197	61.2	125	38.8			
Female	19	52.8	17	47.2			
			ART				
Age (years)					2.665	2	0.273
18-30	53	56.4	41	43.6			
31-50	134	63.8	76	36.2			
51-70	29	53.7	25	46.3			
Sample					62.453	3	**0.000***
Plasma	201	69.3	89	30.7			
CSF	9	15.3	50	84.7			
Vitreous fluid	3	100.0	0	0.0			
Tissue	3	50.0	3	50.0			
ART status					11.863	3	**0.007***
ART naïve	94	68.1	44	31.9			
ART default >3 month	75	61.0	48	39.0			
ART <1 month	15	62.5	9	37.5			
ART ≥ 1 month	32	43.8	41	56.2			
Clinical presentation					53.651	6	**0.000***
Pneumonitis	100	71.9	39	28.1			
Gastro/enterocolitis	65	71.4	26	28.6			
Encephalitis	26	30.6	59	69.4			
Retinitis	12	41.4	17	58.6			
Disseminated	6	100.0	0	0.0			
Bone marrow disease	4	80.0	1	20.0			
Hepatobiliary disease	3	100.0	0	0.0			
CD4 count (cells/μl)					10.376	2	**0.005***
0-100	181	64.2	101	35.8			
101-200	22	53.7	19	46.3			
>200	13	37.1	22	62.9			
HIV viral load (log10)					12.670	3	**0.005***
0-1.3	9	30.0	21	70.0			
1.4-3.0	41	64.1	23	35.9			
3.1-5.0	52	61.9	32	38.1			
5.1-7.0	114	63.3	66	36.7			
Other co-infections					4.392	2	0.107
Yes	150	60.0	100	40.0			
No	62	64.6	34	35.4			
Unsure	4	33.3	8	66.7			
30-days outcome					10.782	1	**0.002***
Alive	183	57.4	136	42.6			
Died	33	84.6	6	15.4			

ART=Antiretroviral therapy, CSF: cerebrospinal fluid, *$p<0.05$* was taken as significant

tested for CMV that may not represent the actual CMV invasion of the central nervous system. Nevertheless, all (100%) patients suspected of disseminated CMV disease had CMV DNA detected in their plasma.

HIV and CMV co-infection associated factors

A chi-square and Fisher's exact test were used to determine the association between the CMV infection and observed background factors in this study, including gender, age, ART status, and other concurrent opportunistic infection. In univariate analysis, a significant association ($p<0.05$) was seen between ART status and CMV infection, where a higher proportion of CMV-infected patients demonstrated in ART naïve and prolonged ART defaulter group, as compared to HIV patients on ART (TABLE 3).

Significant associations ($p<0.01$) were also demonstrated between HIV laboratory biomarkers; CD4 cell count and HIV viral load, with CMV infection when tested individually. A high proportion (64.2%) of HIV patients with a CD4 cell count less or equal to 100 cell/µl were found co-infected with CMV, with a reduction to almost half of the proportion (37.1%) in those with a CD4 cell count more than 200 cells/µl (TABLE 3). The mean CD4 cells in CMV infected group was 50 cells/µl (95% CI: 41.5-59.1), while the mean CD4 cells in CMV non-infected group was higher, 92 cells/µl (95% CI: 74.3-110.5). Meanwhile, a marked increment of more than two-fold was demonstrated in the prevalence of CMV infection in HIV patients with HIV viral load of more than 1.4 log10 when compared to those in stable virologic suppression, HIV viral load < 1.3 log10 (TABLE 3). The mean HIV viral load in the infected group was also found to be higher than the mean HIV viral load in the CMV non-infected group: 618,627 IU/ml (95% CI: 427,299-809,955), and 473,318 IU/ml (95% CI: 297,242-649,393) respectively. A significantly higher proportion of HIV patients in this study (84.6%) that succumbed to death were found co-infected with CMV, leaving only 15.4% who were CMV non-infected (*p=0.002*) (TABLE 3). This data, however, included all causes of mortality throughout the study.

In the final multivariate analysis, only CD4 cell count showed a significant association with CMV infection independent of HIV viral load and ART status. The regression model revealed that decreased CD4 cell count increases the likelihood of CMV infection ($p=0.01$), whereas both ART status and HIV viral load showed no significant association (TABLE 4).

Ganciclovir-resistant CMV testing

Out of 216 samples with CMV DNA detected, 68 samples were re-extracted to proceed with genotypic detection of *UL97* mutation for ganciclovir-resistant testing. These samples were chosen based on the initial quantitative CMV DNA viral load of more than 1,000 copies/ml, with a sufficient volume of equal to or more than 400 µl, and were stored for less than a year at -70 °C. The selection criteria ensure that only the archived samples with preserved DNA quality were continued with the PCR HRM. Despite this, 23 samples showed no amplification during the PCR and 5 samples had late amplification (CT>39). 40/68 (58.8%) samples completed the HRM, and the results showed that 36/40 (90%) were wild type, 2/40 (2.5%) were inconclusive, and 2/40 (2.5%) were M460I/M460V mutation, suggestive of ganciclovir resistance. The melting curves of the wild type and the sample with suspected M460I/M460V mutation are shown in FIG 2.

The two inconclusive samples on HRM had high initial CMV DNA quantitative viral copies that ranged from 8,559 to 354,640 IU/

TABLE 4: Multivariate analysis of CMV and HIV co-infection associated factors

Variables	B	S.E.	*p-value*	AOR	95% C.I.	
ART status						
ART naive	.647	.355	.069	1.909	.952	3.828
ART default >3 month	.234	.353	.508	1.263	.633	2.521
ART <1 month	.504	.517	.330	1.655	.600	4.561
ART >= 1 month				1		
CD4 count (cells/µl)	-.006	.002	**.001***	.994	.991	.998
HIV viral load (log10)	-.062	.078	.428	.940	.806	1.096

**p<0.05* was taken as significant, B: beta coefficient, SE: standard error, AOR: adjusted odd ratio, C.I.: confidence interval.

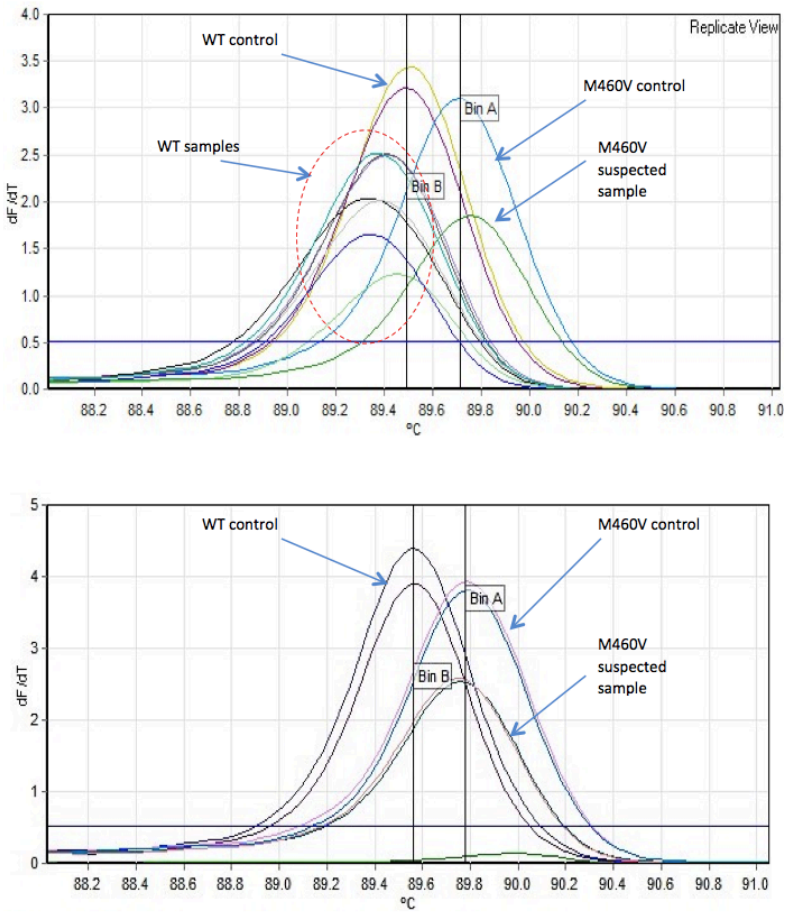

FIG. 2 Melting curves of CMV wild type and M460V mutation. WT: Wild type, Bin B: Melt temperature of Wild type CMV, Bin A: Melt temperature of M460V mutation CMV

ml. Still, the subsequent PCR HRM, on the contrary, showed a high cycle threshold (CT) of 38-39, indicating a low viral copy. This finding could suggest the possibility of DNA degradation during the storage, which results in lower viral nucleic acid, thus affecting the validity of the HRM result. Sanger sequencing was performed directly on the PCR products of the above inconclusive samples, as well as six wild-type samples and two mutant samples, to verify the HRM findings. Image of the PCR products of several samples proceeded with sequencing can be visualised in FIG 3. The subsequent sequencing analysis of these samples showed no M460I and/or M460V mutation detected, therefore confirming that there was no ganciclovir-resistant CMV strain identified in this study. The two samples suspected of *UL97* mutation from the HRM testing showed no M460V/I or H520Q mutation detected, but new mutations, N407H and T409K, were identified. Both mutations have not been reported to be associated with ganciclovir resistance. The results of the sequencing analysis are summarised in TABLE 5.

DISCUSSION

CMV infection in HIV: prevalence and association

In this study, the prevalence of CMV infection in HIV patients was 60.3%, higher than studies in other countries that used comparable molecular methods, such as Thailand and China, with a prevalence of 26.3% and 12.8%, respectively.[25,26] The likely reason for this difference was the approach taken in the study, where only HIV patients who presented with signs and symptoms of CMV disease were recruited regardless of the patient's immune status and/or ART status. Hence, the prevalence in this study was denoted as the prevalence of CMV infection in HIV patients with clinical suspicion of CMV

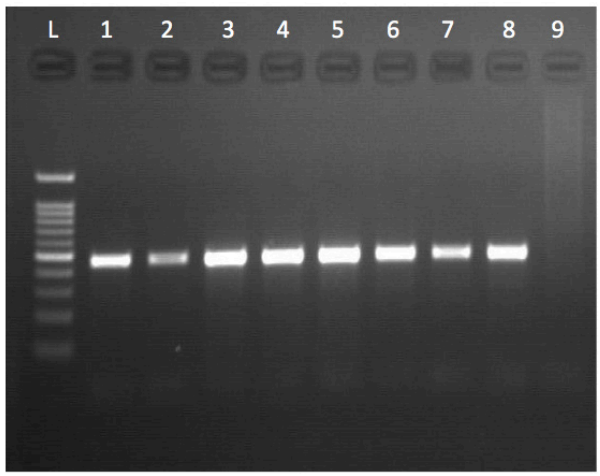

FIG. 3 Image of PCR products from seven CMV positive samples showing amplification of UL97 protein on agarose gel, visualised under UV light. Lane L: 100 bp DNA molecular weight ladder; Lane 1: UL97 positive control; Lane 2-8: CMV positive samples; Lane 9: Negative control.

TABLE 5: Summary of high-resolution melting (HRM) and sequencing analysis

Sample	HRM	In-silico analysis* for M460I/M460V mutation	NCBI GenBank (BLAST)
1	Wild type	No M460I/M460Vdeteced	Identical to human betaherpesvirus 5 H-247 phosphotransferase (UL97) gene. partial cds (KY407403.1)
2	Wild type	No M460I/M460Vdeteced	Identical to human betaherpesvirus 5 H-247 phosphotransferase (UL97) gene. partial cds (KY407403.1)
3	Wild type	No M460I/M460Vdeteced	Identical to Human betaherpesvirus 5 strain SYD-SCT1, complete genome (MT044485.1)
4	Wild type	No M460I/M460Vdeteced	Identical to Human betaherpesvirus 5 strain NL/Rot2/Urine/2012, partial genome (KT726941.2)
5	Wild type	No M460I/M460Vdeteced	Identical to Human betaherpesvirus 5 strain NL/Rot2/Urine/2012, partial genome (KT726941.2)
6	Wild type	No M460I/M460Vdeteced	Identical to human betaherpesvirus 5 H-247 phosphotransferase (UL97) gene. partial cds (KY407403.1)
7	Inconclusive	No M460I/M460Vdeteced	Identical to Human betaherpesvirus 5 strain SYD-SCT1 complete genome (MT044485.1)
8	Inconclusive	No M460I/M460Vdeteced	Identical to Human betaherpesvirus 5 strain HANSCTR13 complete genome (KY490088.1)
9	M460V mutation suspected	N407H and T409K mutations detected. No M460I/M460V detected	Identical to Human betaherpesvirus 5 strain SYD-SCT1 complete genome (MT044485)
10	M460V mutation suspected	N407H and T409K mutations detected. No M460I/M460V-deteced	Identical to Human betaherpesvirus 5 strain NL/Rot2/Urine/2012.partial genome (KT726941.2)

*https://www.informatik.uni-ulm.de/ni/staff/HKestler/hcmv/

disease. Whereas in both aforementioned studies, the study population were HIV patients upon initiation of ART, irrespective of the clinical presentation.

Unlike earlier literature that described retinitis as the most common clinical presentation, comprising >80% of total CMV cases in HIV patients[6], our study found pneumonitis was the leading presentation which accounted for 46.3%, followed by GIT infection, 30.1%. Retinitis was lower in the list with only 5.6% of total cases. Similarly, Perello *et al.* (2019) in their recent study revealed a predominant CMV systemic and lung infections, with nil CMV retinitis case.[5] These concurrent findings could imply a shift in the CMV clinical presentation trend from retinitis to pulmonary or systemic infection in recent years. In a separate study employing histology method, gastrointestinal disease was the most encountered CMV end-organ disease, which comprised two-thirds of all HIV patients, and retinitis was only observed in one-third of the total cases.[27]

The small overall prevalence (3.34%, 12/358) of CMV retinitis with both confirmatory ophthalmological assessment and CMV DNA detection in vitreous fluid could be contributed by the early ART initiation irrespective of CD4 count or World Health Organization (WHO) classification of clinical stages. This prompt ART initiation strategy was practiced as recommended by WHO Consolidated Guidelines on the Use of Antiretroviral Drugs for Treating and Preventing HIV Infection, 2016.[28] This finding is supported by a 6-year observational study in Singapore that demonstrated marked reduction in CMV retinitis incidences with early ART initiation at HIV diagnosis.[29]

Mycobacterium tuberculosis (MTB) infection is the main opportunistic illness in HIV patients in Asia and Africa, contributing to a quarter of all AIDS-related deaths.[30] It was also shown to be the most common concurrent infection in this study. A direct association between CMV infection and tuberculosis specifically in the HIV population is not yet well described. A cohort study in Cape Town, South Africa discovered that infants who have CMV during their first year of life are at a greater risk of acquiring TB disease later in life.[31] However, the study focused on the paediatric population and might not directly signify the adult HIV population.

CMV is also known to predispose immunocompromised patients to invasive fungal infections (IFI), and these dual infections confer even worse survival outcomes.[32] Furthermore, regardless of the co-existence of CMV disease, CMV viraemia alone was also significantly associated with a higher risk of invasive fungal disease.[33] This explains the high number of penicilliosis, invasive candidiasis, PCP and other fungal infections in CMV co-infected HIV patients in this study. These findings also emphasise the importance of screening for the presence of other IFI in patients with CMV viraemia, to accelerate diagnosis and antifungal initiation.

Significant associations ($p<0.05$) were also demonstrated between CMV infections and HIV viral load, CD4 cell counts and ART status when tested individually. However, in the final multivariate analysis, only CD4 cell count showed a significant association, where the value was inversely correlated with CMV infection. A decrease in the CD4 cell count would increase the likelihood of CMV infection regardless of HIV viral load and ART status. Grønborg *et al*. in their review paper discovered a similar association between low CD4 cell count and CMV infection, although they did not discuss the association with HIV viral load.[34] Another observational study stated that the prevalence of CMV increased among HIV patients with low CD4 count (<100 cells/µl), while no association was found between CMV infection and the timing of first-line ART.[26] The majority of HIV patients in this study who were co-infected with CMV were not on ART. The association of CD4 count and CMV infection highlights the importance of immune recovery in preventing CMV infection.

A high proportion of HIV patients with CMV co-infection was seen among the non-survival group compared to the survival group. Although the attributed causes of death were unknown, the possibility of CMV infection as the associated factor should not be overlooked. CMV viraemia was a strong predictor of CMV end-organ disease and death, and the risk proportionately increases with CMV viral load.[35] This finding was somewhat conflicting with an earlier randomised clinical trial by Wohl *et al*. that demonstrated a low rate of CMV disease development among CMV-infected HIV patients, even with persistently low CD4 count.[36] Both studies, however, demonstrated that pre-emptive anti-CMV therapy did not have significant benefits for HIV patients, particularly in preventing CMV disease progression.[35,36] In a separate study, Mayaphi *et al*. also discovered that the mortality increased proportionayely with CMV viral

load, but interestingly, pre-emptive ganciclovir administration was found to be associated with better survival outcome.[37] The same study also suggested that a CMV viral load ≥1,000 copies/ml be the cut-off value to initiate pre-emptive antiviral therapy.[37] The importance of ART was also highlighted recently, as it can effectively restore immunity in HIV patients, reduce CMV viraemia, and prevent the development of CMV end-organ diseases.[38]

CMV and drug resistance

The main mechanism of ganciclovir resistance in CMV is the mutation in the *UL97* gene resulting in diminution of ganciclovir phosphorylation to an active form, and thus unable to exert its antiviral effect.[39,40] Another mechanism is by mutation in the *UL54* gene, altering the viral DNA polymerase to be less inhibited by the wide spectrum of antiviral including ganciclovir, cidofovir and foscarnet.[39,41] Drug-resistant CMV can be detected either via the 'gold standard' phenotypic method or by genotypic analysis. The phenotypic approach is performed by determining a specific concentration of antiviral able to inhibit a standardised amount of CMV growth in a cell culture, and the commonly used method was the plaque reduction assay (PRA).[42] Genotypic diagnosis is attained by looking at the gene mutation(s) that confer resistance to the specific antivirals and the frequently described methods were: i) PCR, with melting curve analysis and/or hybridization probes, and ii) UL97 protein kinase sequencing.[14] Genotypic method is more feasible and favoured over phenotypic testing due to its markedly faster turnaround time, and the results were >90% in an agreement with phenotypic assessment.[16]

Prolonged antiviral exposure, underlying immunosuppression, insufficient antiviral delivery and low activity on the affected site are the risk factors of drug-resistant CMV.[43] Profound immunosuppression with CD4 count <50 cells/μl was a crucial factor of drug-resistant CMV development in HIV patients, with an incidence rate >20% within a year.[42] In this study, findings from HRM and confirmatory genomic analysis of the selected clinical specimens concluded that the CMV strains detected in this centre were wild type, and no M460V or M460I mutations were found. The newly discovered N407H and T409K mutations in the UL97 mutant samples prove the assay's sensitivity to detect genetic variance. Both mutations have yet to be reported to associate with ganciclovir resistance. The finding is likely attributed to the small proportion of CMV co-infected HIV patients who required treatment with ganciclovir, and none among those who received treatment was treated for more than 3 weeks. This is supported by the fact that the development of drug-resistant CMV is predicted to increase by 3-fold in patients exposed to 21 days of antiviral therapy, while reversal of the prevalent wild type strain occurs upon early cessation of the treatment.[44]

Strasfeld & Chou added, a suspicion of CMV drug resistance should be raised when there is a high or rising CMV viral load or worsening CMV illness despite an acceptable course of at least 2 weeks of antiviral medication.[40] Hence, follow-up assessments and repeated CMV drug resistant testing after antiviral therapy courses are suggested in future studies as it could provide more assertive information on drug-resistant emergence in one locality. A continuous surveillance on drug-resistant CMV is also of vital importance as it is linked to a high mortality rate, increased allograft failure in solid-organ transplant recipients, and increased therapeutic (i.e foscarnet) related nephrotoxicity.[45]

HRM is a fast, cheap and simple post-PCR analysis method used for recognising genetic variation in nucleic acid sequences based on differences in melting curve shape or melting temperature (Tm) of the amplicons.[46] HRM is being utilised for analysis of genetic mutations in bacterial and viral pathogens that might have impacts on pathogenicity or drug susceptibility.[47,48] Applying the concept, this study used HRM PCR to detect UL97 mutations namely the M460V/M460I that will indicate ganciclovir-resistant CMV.

In this study, HRM PCR showed a small variance in melting temperature (Tm) between wild type and mutation controls, 0.23°C which caused difficulty in interpreting the results and contributed to the inconclusive and false positive (mutation). This is possibly due to the large size of the amplicon (470 bp) analysed in the study. A large amplicon (greater than 300-400bp) declines the capability of HRM diversity assay in detecting individual mutation, especially when other non-specific mutations can also be present along the amplicon.[49] A smaller amplicon is more advantageous in analysis of single nucleotide polymorphism (SNP) as it will give a discrete Tm difference.[50] Besides, a specific labelled probe can be used to improve the detection but the investigation cost will be increased.

Various mutations were identified harbouring

drug-resistance genes in CMV to date. Majority (90%) of the CMV drug-resistance genes located in UL97 protein, and the five most common genetic mutations encompass 80% of all *UL97* mutations are M460V, H520Q, A594V, L595L and C603W.[14] To detect all of these mutations, the genomic analysis should include the location between amino acid (AA) 460 to 603, which constituted in a large amplicon, and HRM is no longer practical. In this study, the primers used in the HRM targeted an amplification of the most common mutation in the codon 460, and Sanger sequencing was also performed directly using the PCR product focusing on the amplified region. Therefore, although this method has been considered as an appropriate CMV drug resistance screening and used in several previous studies, careful interpretation is required as other drug-resistance related gene mutations located in different codons, can be missed. Moreover, *UL54* mutation which confers resistance to wider antivirals, albeit uncommon, would also be missed by this assay.

This study used archived samples that were stored in the freezer after the initial diagnostic CMV PCR. Sample or DNA degradation could be attributed to the unsatisfactory amplification during HRM, although the initial CMV PCR showed a high level of viral DNA. Future studies using fresh samples would ensure the quality of DNA for further molecular analysis.

CONCLUSION

The predominant clinical presentations of CMV-HIV co-infection were found to be pneumonitis and gastrointestinal diseases, followed by retinitis. The majority of HIV patients who succumbed to death were co-infected with CMV. CMV infection in HIV patients was found to be associated with low CD4 cell count, independent of HIV viral load and ART status. However, the majority of patients with CMV-HIV co-infection were not on ART. Despite the high prevalence of CMV infection detected in clinical specimens, the absence of ganciclovir-resistant strain indicates a good sign. However, the possibility of drug resistance by other less common gene mutations located in different codons cannot be ruled out by using the current HRM method. Rapid improvement in HIV patients' immune systems following early initiation of ART could explain the lower requirement for anti-CMV antiviral therapy and possibly reduce the development of drug-resistant CMV. Prospective studies using wide-ranging molecular methods are recommended to investigate further development of drug-resistant CMV in HIV patients who are on different courses and types of antivirals.

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REFERENCES

1. Zuhair M, Smit GSA, Wallis G, *et al*. Estimation of the worldwide seroprevalence of cytomegalovirus: A systematic review and meta-analysis. *Rev Med Virol*. 2019;29(3):1-6.
2. Cunha Ade A, Marin LJ, Aquino VH, Figueiredo LT. Diagnosis of cytomegalovirus infections

by qualitative and quantitative PCR in HIV infected patients. *Rev Inst Med Trop Sao Paulo*. 2002;44(3):127-132.
3. Meyer-Olson D, Schmidt RE, Bollmann BA. Treatment and prevention of cytomegalovirus-associated diseases in HIV-1 infection in the era of HAART. *HIV Ther*. 2010;4(4):413-436.
4. Jabs DA, Martin BK, Forman MS. Mortality Associated with Resistant Cytomegalovirus among Patients with Cytomegalovirus Retinitis and AIDS. *OPHTHA*. 2010;117(1):128-132.e2.
5. Perello R, Vergara A, Monclus E, *et al*. Cytomegalovirus infection in HIV-infected patients in the era of combination antiretroviral therapy. *BMC Infect Dis*. 2019;19(1):1030.
6. Adland E, Klenerman P, Goulder P, Matthews PC. Ongoing burden of disease and mortality from HIV/CMV coinfection in Africa in the antiretroviral therapy era. *Front Microbiol*. 2015;6:1016.
7. Grønborg HL, Jespersen S, Hønge BL, Jensen-Fangel S, Wejse C. Review of cytomegalovirus coinfection in HIV-infected individuals in Africa. *Rev Med Virol*. 2017;27(1):10.1002/rmv.1907.
8. Emery VC. Restimulating interest in cytomegalovirus as a cofactor for HIV infection. *J Infect Dis*. 2015;211(2):169-171.
9. Springer KL, Weinberg A. Cytomegalovirus infection in the era of HAART: fewer reactivations and more immunity. *J Antimicrob Chemother*. 2004;54(3):582-586.
10. Salmon-Céron D, Mazeron MC, Chaput S, *et al*. Plasma cytomegalovirus DNA, pp65 antigenaemia and a low CD4 cell count remain risk factors for cytomegalovirus disease in patients receiving highly active antiretroviral therapy. *AIDS*. 2000;14(8):1041-1049.
11. Zhao M, Zhuo C, Li Q, Liu L. Cytomegalovirus (CMV) infection in HIV/AIDS patients and diagnostic values of CMV-DNA detection across different sample types. *Ann Cardiothorac Surg*. 2020;9(5):2710-2715.
12. Ahmed A. Antiviral treatment of cytomegalovirus infection. *Infect Disord Drug Targets*. 2011;11(5):475-503.
13. Ritchie BM, Barreto JN, Barreto EF, *et al*. Relationship of ganciclovir therapeutic drug monitoring with clinical efficacy and patient safety. *Antimicrob Agents Chemother*. 2019;63(3):1-10.
14. Chevillotte, Meike, *et al*. A new tool linking human cytomegalovirus drug resistance mutations to resistance phenotypes. *Antiviral research,* 2010;85(2): 318-327.
15. Baldanti, F., & Gerna, G. Human cytomegalovirus resistance to antiviral drugs: diagnosis, monitoring and clinical impact. *Journal of Antimicrobial Chemotherapy*, 2003;*52*(3): 324-330.
16. Jabs, Douglas A., *et al*. Detection of ganciclovir resistance in patients with AIDS and cytomegalovirus retinitis: correlation of genotypic methods with viral phenotype and clinical outcome. *Journal of Infectious Diseases*, 2006;193(12): 1728-1737.
17. Castor, Jared, *et al*. Rapid detection directly from patient serum samples of human cytomegalovirus UL97 mutations conferring ganciclovir resistance. *Journal of clinical microbiology,* 2007; 45(8): 2681-2683.
18. Campos AB, Ribeiro J, Boutolleau D, Sousa H. Human cytomegalovirus antiviral drug resistance in hematopoietic stem cell transplantation : current state of the art. *Reviews in medical virology,* 2016;(March):161-182.
19. Humar A, Snydman D; AST Infectious Diseases Community of Practice. Cytomegalovirus in solid organ transplant recipients. *Am J Transplant*. 2009;9 Suppl 4:S78-S86.
20. Ljungman P, Boeckh M, Hirsch HH, *et al*. Definitions of Cytomegalovirus Infection and Disease in Transplant Patients for Use in Clinical Trials. *Clin Infect Dis*. 2017;64(1):87-91.
21. Sahoo MK, Lefterova MI, Yamamoto F, *et al*. Detection of cytomegalovirus drug resistance mutations by next-Generation sequencing. *J Clin Microbiol*. 2013;51(11):3700-3710.
22. Kraft CS, Armstrong WS, Caliendo AM. Interpreting quantitative cytomegalovirus DNA testing: Understanding the laboratory perspective. *Clin Infect Dis*. 2012;54(12):1793-1797.
23. Göhring K, Mikeler E, Jahn G, Hamprecht K. Rapid simultaneous detection by real-time PCR of cytomegalovirus UL97 mutations in codons 460 and 520 conferring ganciclovir resistance. *J Clin Microbiol*. 2006;44(12):4541-4544.
24. Arefinia, N. *Evaluation frequency of Human Cytomegalovirus resistance to ganciclovir (UL97) in patients with HIV, using High Resolution Melting analysis, Kerman, Iran,* 2018*;* (Doctoral dissertation, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran).
25. Durier N, Ananworanich J, Apornpong T, *et al*. Cytomegalovirus Viremia in Thai HIV-Infected Patients on Antiretroviral Therapy : Prevalence and Associated Mortality. *Clinical infectious diseases,* 2013;57:147-155.
26. Jiang B, Su R, Zhang D, Gao L, Ma P, Lu W. Prevalence and Associated Factors of HIV and CMV Co-Infection in Tianjin, China. *J AIDS Clin Res*. 2018;09(05).
27. Caplan MR, Wilson EMP, Schechter M, *et al*. High prevalence of gastrointestinal manifestations among Cytomegalovirus end-organ disease in the combination antiretroviral era. *J Virus Erad*. 2021;7(3):100052.
28. World Health Organization. (2016). Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection: recommendations for a public health approach, 2nd ed. *World Heal Organ https//apps.who.int/iris/handle/10665/208825*. 2016;(2nd edition).
29. Teoh SC, Wang PX, Wong EPY. The epidemiology and incidence of cytomegalovirus retinitis in the HIV population in Singapore over 6 years. *Investig Ophthalmol Vis Sci*. 2012;53(12):7546-7552.
30. Pawlowski A, Jansson M, Sköld M, Rottenberg ME, Källenius G. Tuberculosis and HIV co-infection. *PLoS Pathog*. 2012;8(2).
31. Martinez L, Nicol MP, Wedderburn CJ, *et al*.

Cytomegalovirus acquisition in infancy and the risk of tuberculosis disease in childhood: a longitudinal birth cohort study in Cape Town, South Africa. *Lancet Glob Heal*. 2021;9(12):e1740-e1749.
32. Jorgenson MR, Descourouez JL, Cardinale B, *et al*. Risk of opportunistic infection in kidney transplant recipients with cytomegalovirus infection and associated outcomes. *Transpl Infect Dis*. 2019;21(3):0-1.
33. Yong MK, Slavin MA, Kontoyiannis DP. Invasive fungal disease and cytomegalovirus infection: is there an association? *Curr Opin Infect Dis*. 2018;31(6):481-489.
34. Grønborg HL, Jespersen S, Egedal JH, *et al*. Prevalence and clinical characteristics of CMV coinfection among HIV infected individuals in Guinea-Bissau: A cross-sectional study. *Trop Med Int Heal*. 2018;23(8):896-904.
35. Mattioni S, Pavie J, Porcher R, *et al*. Assessment of the efficacy and safety of pre-emptive anti-cytomegalovirus (CMV) therapy in HIV-infected patients with CMV viraemia. *Int J STD AIDS*. 2015;26(5):306-312.
36. Wohl DA, Kendall MA, Andersen J, *et al*. Low rate of cmv end-organ disease in hiv-infected patients despite low cd4+ cell counts and cmv viremia: Results of ACTG protocol a5030. *HIV Clin Trials*. 2009;10(3):143-152.
37. Mayaphi SH, Brauer M, Morobadi DM, *et al*. Cytomegalovirus viral load kinetics in patients with HIV/AIDS admitted to a medical intensive care unit: A case for pre-emptive therapy. *PLoS One*. 2014;9(4):1-8.
38. Albasanz-Puig A, Suanzes P, Esperalba J, *et al*. Low frequency of cytomegalovirus (CMV) disease despite high prevalence of CMV viraemia in patients with advanced HIV infection: a clinical and immunological 48-week follow-up study. *HIV Med*. 2021;22(8):682-689.
39. Limaye AP. Ganciclovir-resistant cytomegalovirus in organ transplant recipients. *Clin Infect Dis*. 2002;35(7):866-872.
40. Strasfeld L, Chou S. Antiviral drug resistance: Mechanisms and clinical implications. *Infect Dis Clin North Am*. 2010;24(3):809-833.
41. Razonable RR. Drug-resistant cytomegalovirus: Clinical implications of specific mutations. *Curr Opin Organ Transplant*. 2018;23(4):388-394.
42. Lurain, Nell S., and Sunwen Chou. Antiviral drug resistance of human cytomegalovirus. *Clinical microbiology reviews*, 2010;23(4):689-712.
43. Gilbert, C., and G. Boivin. Human cytomegalovirus resistance to antiviral drugs. *Antimicrobial Agents and Chemotherapy*, 2005;49(3):873-883.
44. Emery, Vincent C., and Paul D. Griffiths. Prediction of cytomegalovirus load and resistance patterns after antiviral chemotherapy. *Proceedings of the National Academy of Sciences*, 2000;97(14):8039-8044.
45. Eid, Albert J., *et al*. Emergence of drug-resistant cytomegalovirus in the era of valganciclovir prophylaxis: therapeutic implications and outcomes. *Clinical transplantation*, 2008:162-170.
46. Montgomery JL, Sanford LN, Wittwer CT. High-resolution DNA melting analysis in clinical research and diagnostics. *Expert Rev Mol Diagn*. 2010;10(2):219-240.
47. Lee HK, Lee CK, Loh TP, Tang JWT, Tambyah PA, Koay ESC. High-resolution melting approach to efficient identification and quantification of H275Y mutant influenza H1N1/2009 virus in mixed-virus- population samples. *J Clin Microbiol*. 2011;49(10):3555-3559.
48. Dehbashi S, Tahmasebi H, Sedighi P, Davarian F, Arabestani MR. Development of high-resolution melting curve analysis in rapid detection of vanA gene, Enterococcus faecalis, and Enterococcus faecium from clinical isolates. *Trop Med Health*. 2020;48(1):1-12.
49. Cousins MM, Donnell D, Eshleman SH. Impact of mutation type and amplicon characteristics on genetic diversity measures generated using a high-resolution melting diversity assay. *J Mol Diagnostics*. 2013;15(1):130-137.
50. AB Applied Biosystems. A Guide to High Resolution Melting (HRM) Analysis. Life Technologies Corporation; 2010. Publication 117GU19-020-081740.