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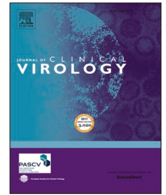

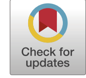

Clinical next-generation sequencing assay combining full-length gene amplification and shotgun sequencing for the detection of CMV drug resistance mutations

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ABSTRACT

Cytomegalovirus (CMV) causes severe systemic and tissue-invasive disease in immunocompromised patients, particularly solid organ and hematopoietic stem cell transplant recipients. While antiviral drugs offer promising efficacy, clinical management is complicated by the high frequency of drug resistance-associated mutations. The most commonly encountered mutations occur in the genes encoding for the drug targets: UL54 (DNA polymerase), UL56 (terminase complex), and UL97 (phosphotransferase), conferring resistance to ganciclovir/cidofovir/foscarnet, letermovir, and ganciclovir/maribavir, respectively. Currently, standard practice for detecting drug resistance is sequencing-based genotypic analysis by commercial reference laboratories with strictly prescribed sample requirements and reporting parameters that can often restrict testing in a highly vulnerable population. In order to circumvent these limitations, we developed a dual-step next-generation sequencing (NGS)-based clinical assay that utilizes full-length gene amplification by long-range PCR followed by shotgun sequencing for mutation analysis. This laboratory-developed test (LDT) achieved satisfactory performance with 96.4% accuracy, 100% precision, and an analytical sensitivity of 300IU/mL with 20% allele frequency. Highlighted by two clinical cases, our NGS LDT was able to provide critical results from patient specimens with viral loads <500IU/mL and volumes <0.5 mL – conditions otherwise unacceptable by reference laboratories. Here, we describe the development and implementation of a robust NGS LDT that offers greater testing flexibility and sensitivity to accommodate a more diverse patient population.

1. Introduction

Cytomegalovirus (CMV) is a significant pathogen in solid organ and hematopoietic stem cell transplant (HSCT) recipients (R), especially those receiving from donors (D) with a mismatched CMV status (i.e., D^{+}/R^{-} or D^{-}/R^{+})[1]. While the risk for fulminant infection and the efficacy of therapy varies between patients, timely diagnostic and monitoring tools are critical for effective management of CMV disease [1]. Moreover, treatment success is challenged by the increasing rates of antiviral resistance specifically in previously-treated individuals [2]. Accordingly, genotyping is often a necessary component of therapeutic monitoring.

Diagnosis of CMV drug-resistance is initially noted by the lack of clinical or virologic response, and is confirmed by target gene sequencing to identify well-characterized mutations that confer resistance [3]. Specifically, resistance to the first-line drug ganciclovir correlates with mutations in the UL97 phosphotransferase, and/or UL54 DNA polymerase. Resistance to cidofovir and foscarnet is associated with mutations in UL54 and resistance to maribavir is associated with mutations in UL97, while resistance to letermovir occur due to mutations in the UL56 terminase complex [4]. Currently, the technical hurdles associated with implementation of next-generation sequencing (NGS)-based assays bar clinical labs from adopting in-house testing, and thus, clinical tests for detection of CMV resistance are commonly limited to reference laboratories with restrictive criteria for acceptable specimens (e.g., 1 mL minimum volume, and ≥500IU/mL minimum viral

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load). In order to circumvent these limitations to offer a more comprehensive service in an institution that provides care to a large population of transplant recipients, we developed and validated a high-throughput laboratory-developed test (LDT). This dual-step NGS-based clinical assay combines full-length gene amplifications of *ul54, ul56,* and *ul97* followed by shotgun sequencing of the pooled amplicons, and streamlined analysis by a commercial software.

2. Methods

2.1. Patients and clinical specimens

This study utilized residual plasma specimens (stored at −70 °C) from CMV viral load testing between 01/2019 and 03/2022. Samples used for this study were collected within the same timeframe (±1 week) as the standard-of-care specimens that were submitted for genotyping to a reference laboratory (Eurofins Viracor, ARUP, and/or Mayo Clinic).

2.2. Primer design

Primers for the full-length amplification of *ul54* and *ul97* were modified based on previously described primers [5]. To increase inclusivity, degenerate bases were used in positions where more than one of the reference sequences differed from the primer sequence (Table 1). Primers for the full-length amplification of *ul56* were designed using 77 unique CMV reference sequences. ≥95% sequence identity with the target sequence and a maximum of 80% identity at off-target sites in all 77 reference sequences was used (Table 1).

2.3. DNA extraction and full-length gene amplification

DNA was extracted from 0.2 mL plasma using the EZ1&2 Virus Mini Kit (Qiagen) and eluted in 60μL of elution buffer. For each gene target, a master mix consisting of Q5 Hot-Start Polymerase 2X Master Mix (New England Biolabs) and gene-specific primers was mixed with 18μL of extracted DNA, with a final primer concentration of 1 μM and a total volume of 50μL. The genes were amplified according to the following conditions: 98°C × 30 s for 1 cycle; 98°C × 10 s, 72°C × 30 s, 72°C × 120 s for 35 cycles; 72°C × 120 s for 1 cycle. After amplification, PCR products were purified using the Monarch PCR & DNA Cleanup Kit (New England Biolabs), and eluted with 30μL of DNA Elution Buffer. Purified PCR products were quantified using the Qubit dsDNA HS kit (Invitrogen).

2.4. Library preparation and shotgun sequencing

Library preparation and NGS were performed as previously described [6]. Briefly, the purified PCR products were tagmented followed by limited-cycle amplification and bead-based purification using the Illumina DNA Prep Kit (Illumina). Libraries were quantified using the Qubit dsDNA HS kit (Invitrogen) and fragment sizes were analyzed using the Agilent DNA 1000 Kit on the Agilent 2100 Bioanalyzer instrument (Agilent). Libraries were pooled and normalized to the appropriate molarity, followed by sequencing on the Illumina MiSeq System.

Table 1
Primer sequences. *ul54* and *ul97* primer sequences were modified based on previously described primers by Sahoo, et al. [5]. *ul56* primer sequences were designed in-house using 77 reference sequences.

Primer		Sequence	Source
ul54	**F1**	CGCGTCGCCGTTGCACGTAG	Modified from Sahoo, et al. [5].
	F2	AGTCCACGCCGCCTCATCTC	
	R1	TCGCAAAAACGGCTACMGACACT	
	R2	TCGTAAGCTGTCAGCCTCTCAC	
ul56	**F1**	ACCGCAGCACCCAGACAGAC	This study
	F2	CACACGCGATAGGGGTACTTGG	
	R1	CAGCGTCTCGTACGTGAGCG	
	R2	GTCAGTCGGCATAGCGAGCG	
ul97	**F1**	GCCACCTTGGTGGACTCGGT	Modified from Sahoo, et al. [5].
	F2	GCAATCYCCGTCACGCCTCTG	
	R1	TGCTGGTRGTGCGCCGAAAG	
	R2	AACCGTCACGTTCCGCGTCC	

2.5. Data analysis

Bioinformatic analysis was performed using the Variant Detection Workflow in the CLC Genomics Workbench (Qiagen). Briefly, sequencing reads were demultiplexed, trimmed for quality, and paired. Paired reads were mapped to *ul54, ul56*, and *ul97* of the reference CMV “Merlin” strain (NC_006273). Codons with known resistance-associated mutations were analyzed for depth and coverage. Non-synonymous nucleic acid differences in these codons were detected using the Low Frequency Variant Detection Workflow and compared to an in-house database of known resistance-associated mutations based on literature review.

2.6. Quality metrics

Sequence data with <50,000 total reads was considered invalid and not analyzed further. For each individual gene, at least 95% of the gene had to be covered by at least 10 reads. Additionally, 30x was required as the minimum depth at the site of mutation, with the mutation present in at least 5 reads. Individual genes that failed quality control metrics were flagged for repeat testing. Additionally, mutations were evaluated based on both a 20% (clinical) and 5% (Research-Use-Only, RUO) allele frequency. Additional review of the sequence data by the Clinical NGS Review Board was required if the minimum quality metrics were not satisfied: (1) if <10% of total reads mapped to the reference genes, (2) any positions in known resistance-associated codons were not covered by at least 30 reads, or (3) average coverage of the target genes was <25x.

3. Results

3.1. Assay design and development

A dual-step CMV genotyping assay combining full-length gene amplification followed by shotgun sequencing of the pooled amplicons was designed to accommodate patient specimens among a wide range of sample volumes and viral loads (Fig. 1). This workflow and chemistry also allow the test to be batched with existing bacterial/fungal whole-genome sequencing (WGS) assays routinely performed in our laboratory for microbial identification and antimicrobial resistance prediction. The compatibility of different NGS assays allow for operational flexibility and cost effectiveness.

To simplify the analytical workflow and reduce technical complexity, analysis is performed on CLC Genomics Workbench using a pre-programmed Variant Detection Workflow with customized QC parameters (Fig. 2, Supplementary Figure 1). The output results from the software (e.g., mapping, coverage, and variant reports) are exported onto a pre-formated Excel worksheet for streamlined result interpretation and reporting.

Additionally, a secondary set of RUO parameters (specifically, a 5% allele frequency cutoff) is included in the analysis to allow for discovery of low-frequency mutations that may be present and clinically significant, but warrant further investigation.

3.2. Accuracy and precision

The accuracy of the assay was determined by concordance with the

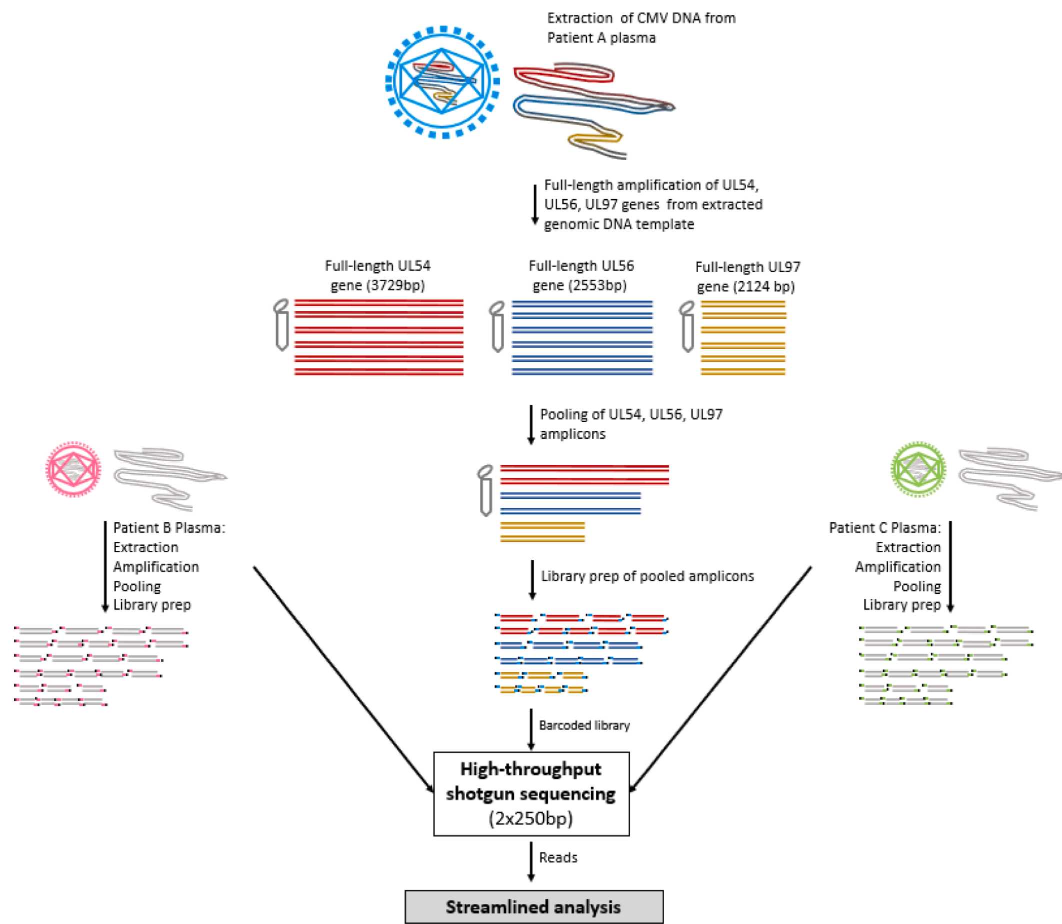

Fig. 1. Schematic of pre-analytical workflow. CMV DNA is extracted from patient plasma and used as template for the full-length amplification of the UL54, UL56, and UL97 genes. The amplicons are pooled for library preparation, and may be combined with other barcoded libraries for sequencing. The reads are analyzed using a streamlined analytical workflow.

standard-of-care method. Drug resistance profiles and resistance mutations were compared. Accuracy of drug resistance profiles were defined as the concordance with the prediction of overall resistance or susceptibility for each drug, while resistance mutation profiles were counted as match or mismatch for each gene. Noted discrepancies were analyzed by patient chart review and comprehensive technical review according to the following criteria: detection of a mutation by the NGS LDT that is discordant with the contemporary standard-of-care sample sent to the reference laboratory was considered true (resolved) if the same result was obtained in a previous sample by the reference method, or can be reproduced using a separate sample by the NGS LDT. Conversely, detection of a mutation that is discordant with the reference method was considered false (discrepant) if the same result was not reproducible by any assay.

The accuracy of drug resistance prediction is 96.4% (Table 2). Among 31 samples tested, 111 drug resistance prediction results were analyzed; 5 discrepant results were noted, with 1 resolved upon review, and 4 remaining (Supplementary Table 1). Notably, in 2/4 discrepant results, the frequencies of the resistant populations were within 1% of the reportable cut-off and would have warranted further investigation by the Clinical NGS Review Board. The accuracy of mutation profile detection is 93.75%. Among the same 31 samples tested above, 80 mutation profiles were analyzed based on the reference result, 6 discrepancies were noted, with 1 resolved upon review, and 5 remaining (Supplementary Table 2). Notably, 1 false susceptible result was due to a resistant minor subpopulation detected below the clinical reportable cut-off but above the RUO cut-off and would have been reviewed by the Clinical NGS Review Board.

The precision of the assay was assessed by testing a clinical specimen with wildtype (WT) CMV and a clinical specimen with drug-resistant CMV in triplicate within the same run, and in triplicate runs. The results produced 100% concordance in both studies (Table 2).

3.3. Analytical sensitivity and specificity

The analytical sensitivity of the NGS LDT was initially determined by testing previously characterized patient specimens across viral loads ranging from below the limit of quantitation (<137IU/mL) up to 1.5 × 10^6IU/mL. 40 samples were included, of which, all 35 samples with viral loads ≥300IU/mL were sequenced with passing quality control metrics (Fig. 3). In contrast, 4/5 samples with viral loads <300IU/mL failed quality control metrics for at least one target gene, and 2/5 failed quality control metrics for all. All 12 samples within $+1\log_{10}$ (300–3,000IU/mL) of the presumptive limit of detection (LoD) of 300IU/mL passed QC metrics for all genes. Accordingly, the minimum viral load required was set at 300IU/mL, with a minimum sample volume of 0.2 mL (Table 2).

To assess the proportional sensitivity of the NGS LDT at detecting mutations at varying viral subpopulations, previously characterized and contrived samples were tested. One specimen with naturally-occurring resistant subpopulation at a low frequency of 2.5% (weighted average) was reliably detected at a rate of 100% (6/6) in dilutions with viral load ≥12,000IU/mL, and thus a calculated resistant subpopulation of approximately ≥300IU/mL (Fig. 4, Supplementary Table 3). Additionally, contrived samples were produced using previously characterized patient samples with resistant and WT CMV strains at varying resistant subpopulation ratios and total viral loads. Consistent with the

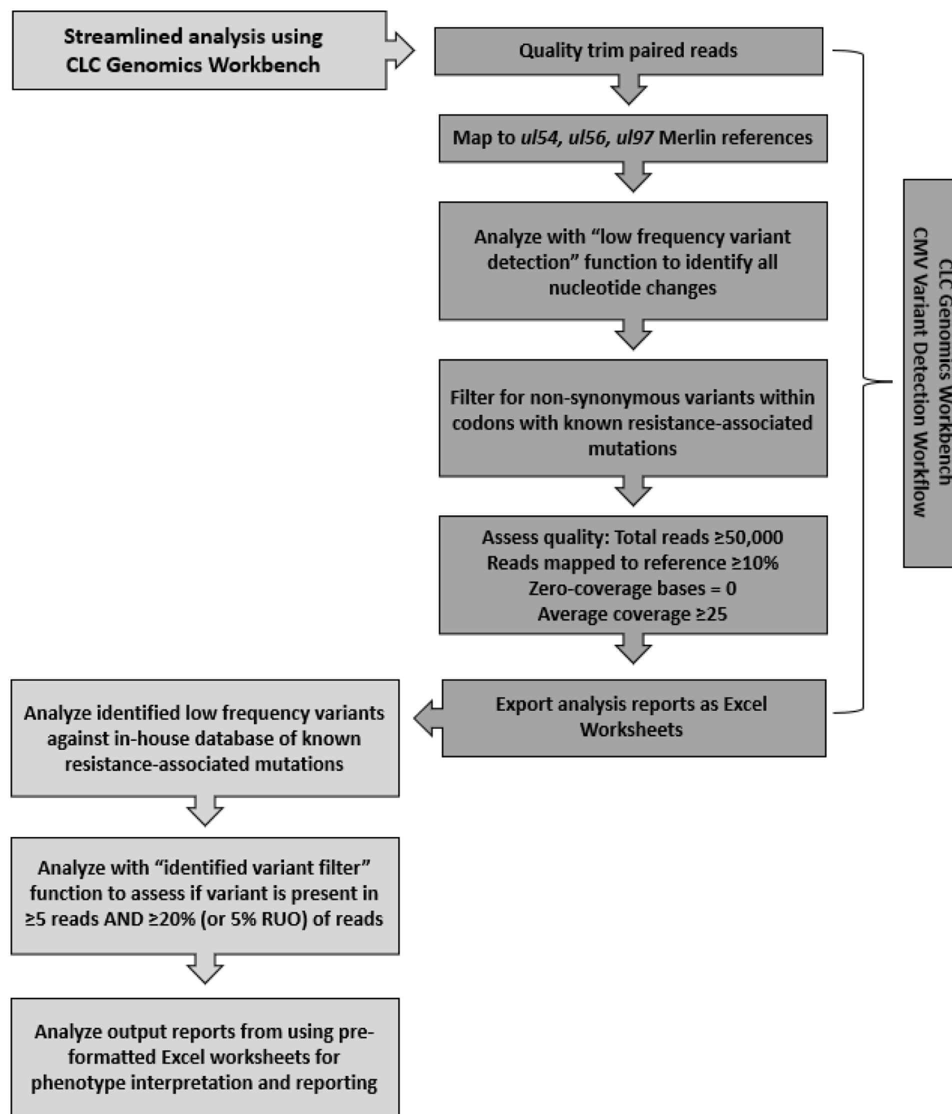

Fig. 2. Overview of analytical workflow. Sequencing reads are imported and paired in CLC Genomics Workbench. The paired reads are analyzed using the CMV Variant Detection workflows, which performs quality trimming, mapping to reference sequences, identifying non-synonymous variants, and assessing the quality of results. All analysis reports from the workflow are exported as Excel Worksheets and analyzed for phenotype interpretation and reporting.

Table 2
Performance Characteristics. Performance of the NGS LDT was tested using contrived and previously characterized patient specimens, and the results were evaluated according to concordance with standard-of-care testing results by a reference laboratory.

Study		Total Results Analyzed	Accurate results	Inaccurate Results	Results
Accuracy	**Drug Resistance**	111	107	4	96.40%
	Mutation Profile	80	75	5	93.75%
Sensitivity		23	22 True Resistance	1 False Susceptibility	95.65%
Specificity		88	85 True Susceptibility	3 False Resistance	96.59%
Analytical Sensitivity		33	33	0	100% at 300IU/mL
Analytical Specificity	**UL54**	1790	1787	3	99.83%
	UL56	306	306	0	100%
	UL97	942	942	0	100%
Precision	**Within run**	3	3	0	Wild-type: 100%
		3	3	0	Resistant: 100%
	Between runs	3	3	0	Wild-type: 100%
		3	3	0	Resistant: 100%

results above, in 100% (4/4) of contrived samples in which the calculated resistant subpopulation was $\geq$300IU/mL, the LDT was able to accurately detect the mutation variant(s) (Fig. 4). Overall, resistant subpopulations were detected in all samples in which they were present above the detectable threshold, with 8/10 tested samples within $+1\log_{10}$ of the LoD. Notably, viral subpopulations between 100 and 300IU/mL were still detected by the NGS LDT in 6/19 samples. Altogether, regardless of population heterogeneity, the CMV resistance NGS

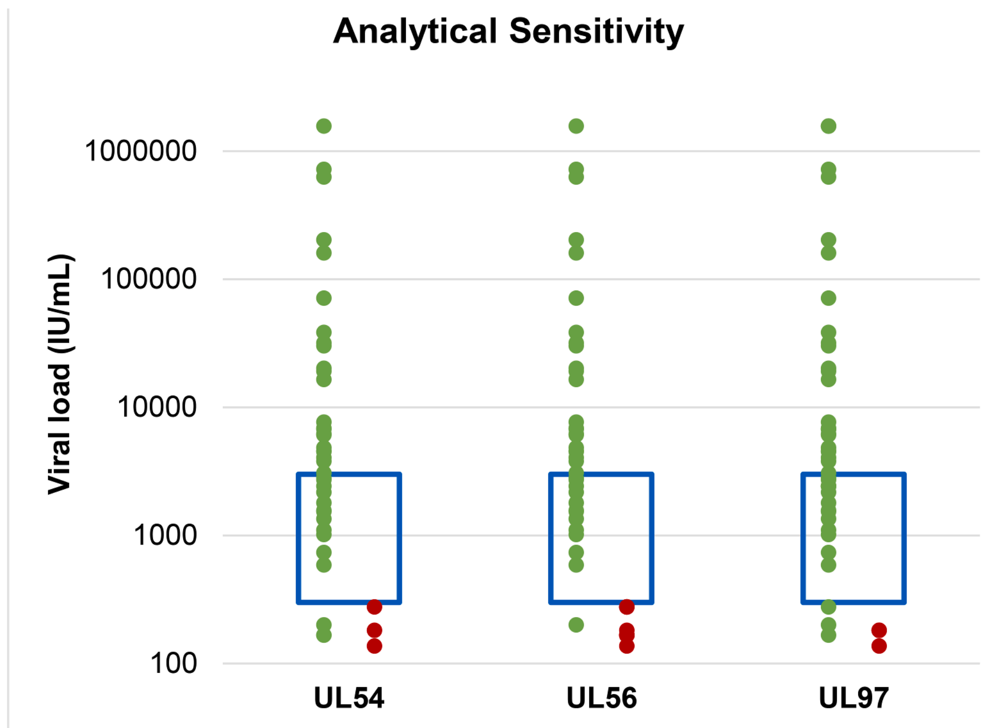

Fig. 3. Determination of analytical sensitivity. Analytical sensitivity was determined as the lowest viral load that provided 100% passing quality metrics. The blue box denotes viral loads from the LoD to $+1\log_{10}$xLoD (300–3,000IU/mL). Samples with passing quality metrics are marked in green, and samples with failed quality metrics are in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

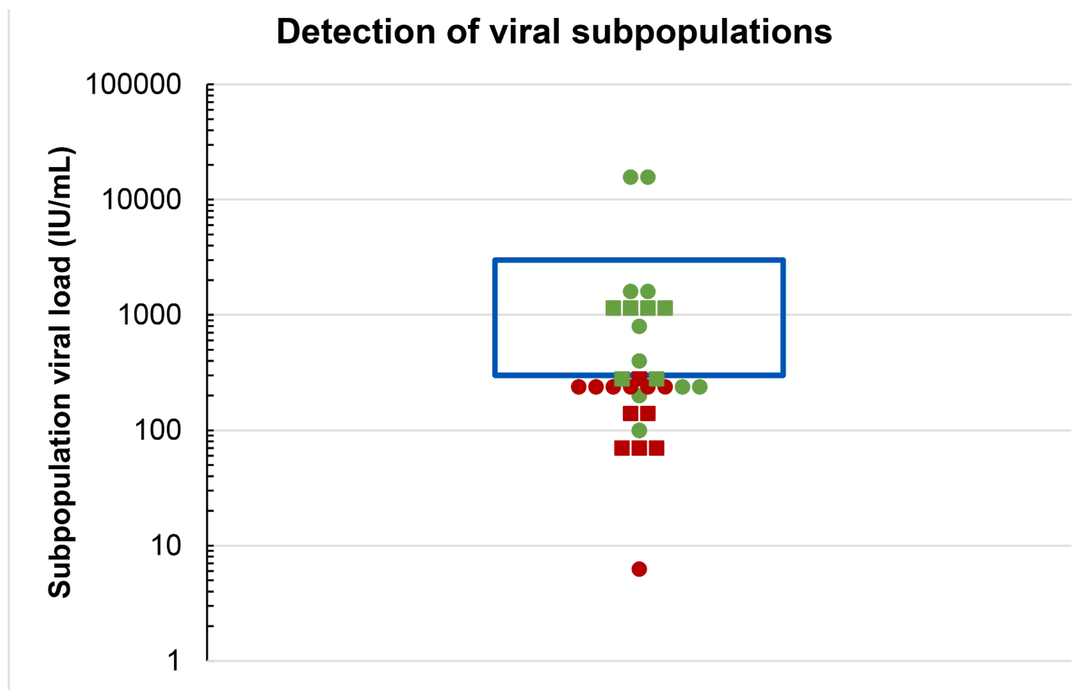

Fig. 4. Determination of proportional sensitivity. Proportional sensitivity was determined as the lowest viral load of the resistant subpopulation that provided 100% passing quality metrics. The blue box denotes viral loads from the LoD to $+1\log_{10}$xLoD (300–3,000IU/mL). Samples with passing quality metrics are marked in green, and samples with failed quality metrics are in red. Natural clinical specimens are marked with circles, and contrived samples are marked with squares. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

LDT achieves acceptable performance with accurate results provided the minor resistant subpopulation remains ≥300IU/mL.

The analytical specificity of the assay was determined by the concordant absence of resistance mutations at each resistance-associated site in the entire gene, for each gene. For instance, there are 17 resistance-associated sites on UL56 among 18 samples for a total of 306 points of concordance. In the same token, there are 58 and 31 resistance-associated sites for UL54 and UL97, respectively. The overall

specificity of drug resistance detection is >99.8% for all 3 target genes (Table 2).

3.4. Utility and clinical application assessment

The primary impetus for developing an in-house test for detecting CMV drug resistance was to allow for a more comprehensive testing capability to accommodate the diverse needs of a high-risk patient population. The clinical utility of our assay is reviewed and highlighted in the following 2 cases:

A 59-year-old lung transplant patient and CMV gastritis presented with fever and hypoxia, which prompted high clinical suspicion for invasive CMV disease. The patient was initially placed on ganciclovir, then switched to valganciclovir twice daily. Throughout the course of the presentation, the antiviral therapy suppressed the viral load to <1,000IU/mL; thus, in both attempts in resistance genotyping by two different reference laboratories, the results returned as indeterminate, citing insufficient viral quantity for testing; thus, use of the in-house CMV resistance LDT was prompted. Using a sample with CMV viral load of 338IU/mL, the NGS LDT was remarkably successful and detected two resistance-associated variants: C603W on UL97, which confers resistance to ganciclovir, and T503I on UL54, which confers resistance to cidofovir/ganciclovir.

In another case, a 57-year-old acute myeloid leukemia patient underwent HSCT. The patient was initially on letermovir for primary CMV prophylaxis until post-transplant screening revealed CMV viremia up to 12,815IU/mL. The patient was placed on ganciclovir therapy, which effectively reduced viral burden to <1000IU/mL by the completion of the first regimen. Subsequent regimens of ganciclovir, however, failed to achieve the same efficacy, and 2 months later, genotyping by a reference laboratory identified the mutation A594P on UL97, confirming ganciclovir resistance. The patient was consequently admitted for foscarnet induction, which reduced the viral load to <500IU/mL. While successfully treated with foscarnet, the anticipated need to replace the patient on prophylactic management prompted the concern for possible letermovir resistance. However, the low viral load prohibited sending for the CMV genotyping test to reference laboratories. CMV genotyping with the NGS LDT was prompted on a residual sample with viral load of 441IU/mL. Again, the NGS LDT performed successfully and confirmed the absence of letermovir resistance-conferring mutations, providing confidence in returning the patient to letermovir-based prophylaxis.

4. Discussion

CMV infection or reactivation is a major concern in transplant recipients as a common consequence of the immunosuppressive drugs intended to mitigate the risks for transplant rejection [7]. Accordingly, effective tools for monitoring CMV infections are critical for maintaining successful outcomes post-transplantation. Currently, the standard-of-care method for detecting antiviral resistance is genotyping of the viral drug targets. Due to the high cost and extensive labor involved, clinical genotyping assays are commonly offered only by reference laboratories with restrictive specimen requirements that are not always feasible with our large transplant population. Thus, we developed a highly sensitive NGS assay that combines long-range PCR with shotgun sequencing. Corroborated by two clinical cases, our NGS LDT was able to perform successfully using residual samples with <0.5 mL plasma volume and <500IU/mL viral load – conditions otherwise unacceptable for send-out testing.

Additionally, our assay includes RUO features such as detection thresholds as low as 5%, and comprehensive variant detection to allow for discovery of minor subpopulations or novel resistance-associated mutations.

One limitation is that our NGS LDT does not include interpretation of identified resistance determinants associated with maribavir resistance due to unavailability of high-quality database. While the NGS LDT will agnostically detect genetic variants in the target genes, mutations linked to possible maribavir resistance will be limited to RUO until more data become available. Additionally, recent in vitro studies have implicated mutations in UL27 as a mechanism to confer resistance to maribavir by compensating for the maribavir-induced inhibition of UL97 function [8]. Our NGS LDT does not currently include the *ul27* gene in its analysis. Lastly, the resistance database employed in our assay was curated and validated through extensive literature review. Moving forward, the database must be updated at least annually to reflect current literature.

In summary, this study provides a detailed protocol and performance characteristics of a highly sensitive and accurate NGS-based clinical test for CMV resistance that utilizes accessible commercial platforms and can detect drug-associated mutations in samples with extremely low viral load. We demonstrated the utility of this test in serving the unmet clinical needs for transplant patients with suspected CMV antiviral resistance, but having very low viral load.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2023.105520.

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