Use of Illumina MiSeq Technology to Detect Drug Resistance Mutations in Human Cytomegalovirus

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Abstract

Background: Cytomegalovirus (CMV) is extremely common with a seroprevalence of 50-80%. In most instances, the virus establishes a lifelong, latent infection with minor sequelae or subclinical manifestations. However, infection in immunocompromised hosts (i.e., transplant recipients) may be associated with severe morbidity and mortality, causing disease in multiple organ systems[1]. Due to the development of antivirals and their use as prophylactic therapy in transplant recipients, the incidence of CMV disease has been reduced. However, prophylactic therapy is believed to have resulted in increased antiviral resistance[2]. Currently, Sanger sequencing of the UL54 and UL97 genes of CMV is considered the gold standard for detection of resistance mutations. This method has limited sensitivity and may not be able to detect minor viral populations with resistance mutations. In this study, we developed a Next Generation Sequencing (NGS) method using the MiSeq platform (Illumina, San Diego, CA) to detect mutations associated with antiviral resistance in CMV and compared the results to traditional Sanger sequencing.

Methods: Long-range PCR primers were designed to target the intergenic sequences of UL54 and UL97 and analyzed with Integrated DNA Technologies (IDT, Coralville, IA) OligoAnalyzer and NCBI Blast to assess analytical specificity. Clinical plasma samples (n=5; 3 resistant, 2 wild type) submitted for routine CMV genotypic testing by Sanger sequencing were also extracted using the GeneJet viral DNA and RNA purification kit (Thermo Scientific, Waltham, MA) and PCR performed using the Applied Biosystems Veriti 96 well thermocycler. The amplified products were quantitated and equimolar concentrations of UL54 and UL97 were pooled. Fifty nanograms of pooled DNA were sheared to roughly 350 bp. Libraries were prepared using the TruSeq nano DNA sample preparation kit (Illumina), indexed, and sequenced on a MiSeq sequencer. Raw FASTQ sequences were demultiplexed and extracted with the Illumina CASAVA program. Sequences were then aligned to the reference CMV Merlin genome (NC_006273.2) with the CLC Bio Genomics workbench v6.0 (Qiagen) and subjected to single nucleotide polymorphism (SNP) and indel detection using CLC Bio Quality and Probability software. The resulting nucleotide-to-amino acid modifications were analyzed using an in-house developed software program. Sequence .BAM files was submitted to Advanced Biological Laboratories and CMV clinical genotyping reports were generated using DeepChek software.

Results: Three samples determined to have CMV with UL97 mutations conferring ganciclovir resistance by Sanger were tested by the NGS method, and the resistance mutations were detected in all 3 samples by NGS. Furthermore, 2 clinical samples determined to harbor wild-type CMV by Sanger were confirmed by NGS. Interestingly, silent mutations (n=190) as well as mutations affecting the amino acid sequence (n=49) were detected among the clinical samples by the NGS method.

Conclusions: The novel NGS method detected mutations conferring ganciclovir resistance in 3 patient samples originally characterized by Sanger sequencing. In addition, mutations of unknown significance were detected and need to be characterized for their potential role in conferring drug resistance. Current studies are underway to assess a larger number of clinical samples, including samples harboring CMV with UL54 mutations. Finally, we hope to assess the ability of the NGS method to detect and differentiate mixed populations of mutant and wild type virus in the same clinical sample.

Objectives

1. Develop and evaluate a sensitive and specific test using MiSeq platform to identify mutations in UL54 and/or UL97 that are responsible for antiviral resistance
2. Compare the Illumina® MiSeq platform to Sanger sequencing for detecting CMV antiviral resistance

Methods

- Long range nested PCR primers for UL54 and UL97 were designed using published sequences[3] and IDT primer design software
- Specificity checked with NCBI BLAST and by performing PCR with HSV 1/2, VZV, and EBV
- Samples analyzed by routine Sanger sequencing requisitioned for analysis by Illumina MiSeq platform
- DNA isolated with Gene Jet viral nucleic acid kit and amplified
- Amplicons quantitated, pooled and sheared to ≈ 350bp
- Libraries indexed and sequenced on MiSeq platform
- Libraries prepared with TruSeq nano DNA sample prep kit (Illumina)
- Raw FASTQ sequences were demultiplexed and extracted by Illumina CASAVA program
- Sequences aligned with CMV Merlin genome (NC_006273.2) with CLC Bio Genomics workbench (Qiagen)
- CLC Bio Quality and Probability software used to detect SNP and indels
- Nucleotide to amino acid modification analyzed using in house software
- Test Reports were also generated with Advanced Biological Laboratories DeepChek software

Figure 1. Study Work Flow

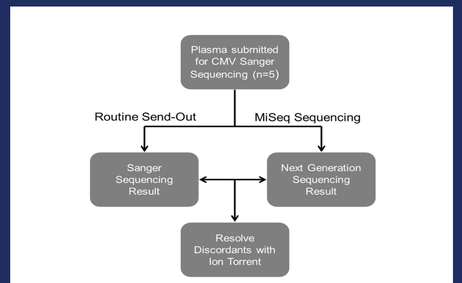

Table 1: Mutants Detected by Each Method

Patient	Source	Sanger Sequencing Result (UL54)	Sanger Sequencing Result (UL97)	MiSeq Result (UL97)	Drug Resistance Conferred	# of "Other" Single Nucleotide Polymorphisms
1	Whole Blood	WT	M 460 I	M 460 I	Ganciclovir	8
2	Serum	WT	WT	WT	None	9
3	Plasma	WT	WT	WT	None	10
4	Fluid	WT	L 595 S	A 594 V, L 595 S	Ganciclovir	6
5	Plasma	WT	H 520 Q	H 520 Q, C 603 R	Ganciclovir	16

Table 1. Results of Sanger sequencing and MiSeq next-generation sequencing on patient samples. MiSeq was able to detect the same mutations as the standard laboratory method. The MiSeq platform was also able to detect additional drug resistance mutants in patients 4 and 5. Additional mutations of unknown significance were found in each of the patient samples.

Figure 2. Example of CMV Drug Resistance Genotype Patient Report

A.

CMV inhibitor

	Algorithm	20.00%	5.00%	1.00%
Cidofovir	Viracor-IBT	S	S	S
	CMV Literature Review	S	S	S
Foscamet	Viracor-IBT	S	S	S
	CMV Literature Review	S	S	S
Ganciclovir	Viracor-IBT	R	R	R
	CMV Literature Review	R	R	R
Maribavir	Viracor-IBT	NA	NA	NA
	CMV Literature Review	S	S	S

	Viracor-IBT	CMV Literature Review
S	S	S
I	I	I
R	R	R

B.

CMV UL97 mutations

Position	Mutation	20.00%	5.00%	1.00%	Prevalence %
436	L			✓	1.47
443	T			✓	1.88
444	E			✓	1.08
445	L			✓	1.28
450	R			✓	1.04
454	R			✓	1.04
455	L			✓	1.09
460	I	✓	✓	✓	69.88
462	A			✓	1.14

Fig 2. .bam files were submitted to Advanced Biological Laboratories (ABL) for analysis by DeepChek software. The report included an initial page that noted the drug resistance mutations that were tested, where the information regarding the phenotype associated with genotype is found and finally what percentage of the reads were susceptible or resistant to a given drug (A.). Subsequent pages consists of in depth notations of the position of the mutant codon, what mutation occurred and the prevalence of the mutation (B.).

Discussion/Future Directions

- The Illumina MiSeq Next-Generation Sequencing platform is capable of detecting drug resistance mutations that Sanger sequencing does not detect.
- The preceding study serves as a proof of principle for using the Illumina MiSeq platform for genotypic resistance testing.
- Larger sample numbers are needed to adequately asses the capability of the MiSeq platform.
- Further experiments include evaluating UL54 mutations and examining mixed virus populations.

References

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