In transplantation, detecting CMV antiviral resistance

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February 2021—Eighteen months after introducing a next-generation sequencing assay to detect CMV antiviral resistance in the transplant population, Matthew Binnicker, PhD, D(ABMM), in an AMP presentation, shared a patient's case and his laboratory's broader experience.

The diagnostic advantages of the NGS assay are earlier detection of resistance and better management of cytomegalovirus in transplant patients, said Dr. Binnicker, director of clinical virology and vice chair of practice, Department of Laboratory Medicine and Pathology, Mayo Clinic, in the virtual AMP2020 presentation in November.

As the number of transplants rises in the United States, he said, "we're going to see an increasing number of individuals who are on high levels of immunosuppressive medications, and this puts them at an increased risk of infectious diseases like CMV."

Dr. Binnicker, who is also professor of laboratory medicine and pathology, discussed the Mayo Clinic case of a 67-year-old female patient with a history of polycystic kidney disease, who underwent a living, unrelated-donor renal transplant. Her serostatus was determined pre-transplantation to be a mismatch for CMV IgG: "The donor was seropositive, but the recipient was seronegative." The patient's Epstein-Barr virus serostatus was donor-positive, recipient-positive.

The patient presented five months post-surgery with elevated liver enzymes and described two days of diarrhea leading up to her presentation. Because of these clinical manifestations and the patient's serostatus pre-surgery, the team ordered a CMV viral load on a plasma sample, which was reported as elevated at 130,000 IU/mL—"definitely a result that would be concerning, along with these clinical manifestations, for possibly a CMV disease in the post-transplant setting," Dr. Binnicker said.

The patient was started on ganciclovir, and a test performed the following week found an elevated CMV viral load of more than 1 million IU/mL. "It's not too concerning to see a viral load increase even after an antiviral has started. We need to wait at least a few weeks before we begin thinking about resistance or changing management," Dr. Binnicker said.

The patient's viral load showed a "nice response" over the next eight to 10 weeks, plateauing between 100 and 1,000 IU/mL, he said. At weeks 11 and 12 post-presentation, however, the patient's viral loads rebounded to high levels while on ganciclovir. "We began to have significant concerns that resistance had developed in this individual."

The clinical manifestations of CMV "can be nonspecific and difficult for health care teams to diagnose and manage, which makes diagnostic testing so critically important," Dr. Binnicker said. CMV syndrome, which can include fever and elevated neutrophil counts, is a common manifestation. "When an individual presents with that category of CMV syndrome, testing is important."

Other clinical manifestations are pneumonitis, hepatitis, and gastrointestinal disease with nausea, vomiting, or diarrhea, as was seen in the 67-year-old patient. "In some cases, central nervous system disease can also occur," he said.

Dr. Binnicker

Prolonged exposure to an antiviral drug (median is five months), lack of immunity pre-transplant (donor-seropositive/recipient-seronegative), on a regimen of a strong immunosuppressive therapy, and inadequate antiviral drug delivery are risk factors for CMV antiviral resistance, Dr. Binnicker said. The incidence of antiviral resistance in CMV depends on the type of transplant and the patient, he said, but has been measured at between five and 12 percent in the solid organ transplant population and, depending on the study and population tested, between 1.7 and 14.5 percent among stem cell transplant recipients. Higher incidence rates are seen in recipients of lung (18 percent) and intestinal or multiorgan (31 percent) transplants.

Specific mutations in two CMV genes have been associated with antiviral resistance, Dr. Binnicker said.

"The first and most common gene in which we see mutations associated with antiviral resistance is *UL97*," which encodes for a kinase required for activation of ganciclovir and valganciclovir, one of the most common classes of drugs used to treat CMV in transplant patients, he said. Specific mutations occurring within *UL97* "can prevent the phosphorylation or activation of those drugs."

Mutations in the CMV gene *UL54*, which encodes for a DNA polymerase, are less common but can have a more severe outcome. These mutations are associated with resistance to the antiviral therapies cidofovir and foscarnet, in addition to ganciclovir and valganciclovir. "In some cases, there is cross-resistance to multiple drugs where we have fewer options for treatment in those patients," Dr. Binnicker said. The mutation D301N, for example, can cause cross-resistance to ganciclovir and cidofovir.

"There are new classes of drugs coming out and we're discovering there can be mutations in other CMV genes like *UL56*, or less commonly in *UL89* and *UL51*, that can result in resistance to newer classes of drugs, such as letermovir," Dr. Binnicker said. The list of mutations in *UL56* is growing as more sequencing is done.

Persistent or recurrent high viral loads during prolonged (greater than six weeks) antiviral therapy raise suspicion for antiviral resistance. "As in the case highlighted, that individual had been on ganciclovir therapy for eight to 10 weeks and then showed a rise in their viral loads," Dr. Binnicker said. A patient with viral loads that plateau at a high level, despite being on therapy, "can lead us down the road of thinking about a resistant population arising."

Dr. Binnicker and colleagues at Mayo Clinic in Rochester developed a next-generation sequencing method for detecting resistance in CMV. The method, implemented for routine use in May 2019, requires a plasma sample from an individual who has had a viral load performed. "We've required that the viral load be 500 IU/mL or higher," he said.

Fig. 1. Sequence analysis

CMV *UL54* mutations				
Position	Position coverage (40×)	Mutation	Prevalence %	Mutation coverage (20×)
24	11008	S → L	94.52	10405
898	5697	N → D	98.74	5625
1108	5961	A → T	98.86	5893

CMV *UL97* mutations				
Position	Position coverage (40×)	Mutation	Prevalence %	Mutation coverage (20×)
19	27964	Q → E	97.39	27235
75	21047	T → A	99.36	20913
108	21097	S → N	98.33	20744
126	20857	Q → L	98.68	20582
460	**21450**	**M → V**	**21.03**	**4511**
594	**20862**	**A → V**	**18.56**	**3872**
599	**18707**	**K → del**	**7.93**	**1484**
603	**22384**	**C → W**	**37.56**	**8408**

Mutations of interest based on CMV variant database (Bold red text: ≥15%); (Bold blue text: <15% but ≥5%)

"Once we have that sample, we send it through automated extraction to recover the DNA from the sample. Then we perform two separate PCRs on the viral nucleic acid" and amplify the entire *UL97* and *UL54* genes.

"We run a gel and check that the amplicon is the right size. Then we mix the products together, the *UL54* and *UL97* amplified material, into a standardized concentration and send it to our core next-generation sequencing facility, where the sequence is generated on the Illumina MiSeq."

Once the sequence is completed, Dr. Binnicker's team uploads the sequence data to Advanced Biological Laboratories, which has developed a bioinformatics analysis software program. "The software interrogates that sequence file and compares it with a reference database—wild-type CMV that lacks resistance-associated mutations."

Within 30 minutes, he and his team receive a report from ABL summarizing whether there is predicted resistance or susceptibility to cidofovir, foscarnet, or ganciclovir (**Fig. 1**). "We can dive into the data more and look at the specific mutations that were identified in comparison with the reference database," Dr. Binnicker said.

The CMV mutation analysis report lists all mutations identified in *UL54* and *UL97* and distinguishes the mutations associated with antiviral resistance, which are further distinguished by red text if present at a threshold of 15 percent or higher and by blue text if they are at low levels, between five and 15 percent of the reads.

"We wanted to call out those low-level mutations, those coming up between five and 15 percent, for monitoring and discovery purposes," Dr. Binnicker said. "If we see in a patient that a resistance mutation is present at, say, 7.93 percent, we wanted to call that mutation out so that we can track it over time. We can see then how that viral mutation emerges or evolves as that patient is on antiviral therapy."

Dr. Binnicker and colleagues conducted a multiple-month validation of the test to ensure its accuracy, during which time they compared it with Sanger sequencing. They defined the limit of detection, confirming the lowest viral load that produced reproducible and accurate sequencing data.

"We were interested in assessing whether the method could identify whether mixed viral populations were present in the sample and be able to tell us whether there was a minor subpopulation that could harbor resistance in the presence of a majority wild-type background," he said.

Dr. Binnicker's team also studied analytical specificity and precision using prospective clinical samples. The team found complete agreement between the Sanger and NGS methods for predicting antiviral susceptibility in 11 clinical samples (**Fig. 2**). "We also saw good agreement for resistance calling for the drugs ganciclovir, cidofovir, and foscarnet, so about 97 percent overall agreement between the two platforms at the level of susceptibility and resistance calling."

In one patient sample, the NGS method gave a call of ganciclovir resistance, whereas the Sanger method called it as foscarnet resistance. "We followed up on this with additional sequencing and weren't able to confirm the results of the Sanger method calling out foscarnet resistance, so we felt that the ganciclovir call was most likely accurate in that setting," Dr. Binnicker said.

A deeper dive into the comparison data revealed how well each method performed at specific mutation calling in the *UL97* and *UL54* genes. "The vast majority of mutations detected in our studies showed agreement between the two sequencing methods," he said. Some mutations detected by NGS were not detected by Sanger, "and when we looked at the prevalence of those mutations, they were less than 20 percent in most cases, which is typically the threshold for Sanger to be able to identify those," Dr. Binnicker said. "This likely calls out some increased sensitivity or discriminatory power of next-generation sequencing."

Two samples showed mutations detected by Sanger but not by NGS, but "we weren't able to confirm those with our subsequent sequencing studies," he added.

Fig. 2. CMV NGS: accuracy

	Susceptible	Ganciclovir resistance	Cidofovir resistance	Foscarnet resistance
Number of interpretations by NGS predicting	11	17	1	2
Number of interpretations by Sanger sequencing predicting	11	16	1	3

96.8% overall agreement between NGS and Sanger sequencing for determination of antiviral drug resistance among clinical plasma samples.

Additional validation studies confirmed that the LOD is reproducible and highly accurate at 500 IU/mL.

For mixed viral population studies, Dr. Binnicker and his colleagues took clinical samples with known *UL97* and *UL54* mutations and mixed them in varying ratios. "We were able to detect and differentiate the expected mutations by our next-generation sequencing method," he said. They also wanted to find out how low prevalence a minor subpopulation can be with a mutation in the background of a majority wild type and whether NGS can differentiate them.

The team mixed wild-type CMV with CMV known to harbor various *UL97* or *UL54* mutations at different ratios. "We identified that if the minor subpopulation is present at a level of 15 percent or above, we were able to reproducibly and accurately identify that mutation and report it out," he said.

"One of the fascinating aspects we've discovered as we started to use next-generation sequencing is the ability of the technology to identify low levels or minor subpopulations of the virus that can have emerging resistance mutations," Dr. Binnicker said. "With Sanger, you need to have at least 20 percent of a viral population present with resistance to be able to tease it apart from a wild-type strain that lacks resistance." With NGS, "we can get down to prevalence levels of 10 to 15 percent and be able to discriminate viral populations from each other." Minor subpopulations may emerge to become predominant populations if not caught early.

When it comes to sequence coverage and reads, "Sanger typically falls apart in terms of its quality of sequencing after about 600 to 700 base pairs," he said. NGS can sequence thousands of base pairs, "even the full-length *UL97* and *UL54* genes," with high-quality results.

NGS offers a lower LOD compared with Sanger—500 IU/mL for NGS, "and in some cases even lower"—compared with greater than 1,000 IU/mL for Sanger. Throughput is much higher for NGS compared with Sanger.

In comparing the two sequencing methods, the only advantage found in the Sanger method was financial. "Sanger is definitely less expensive than next-generation sequencing," Dr. Binnicker said, though he predicts NGS costs will decrease and notes the benefits to transplant patients are likely worth the higher cost of NGS. "When we're dealing with complex cases and need to determine early on whether resistance is emerging, next-generation sequencing is a good application in these high-risk individuals."

Since implementing the CMV NGS method for routine testing in May 2019, Dr. Binnicker's laboratory is seeing some level of resistance-associated mutations in 20 to 25 percent of all samples tested. "That speaks to the fact that a lot of times this test is being ordered when there's evidence of treatment failure or suspicion for resistance, and we're definitely seeing a fairly good rate of resistance being detected by the next-generation sequencing method," he said.

By tracking the specific mutations identified using NGS, they have found that the L595S mutation "is the one we have seen with the highest frequency," Dr. Binnicker said. "About 28 percent of all the samples that show resistance—and 4.7 percent of all samples tested—have that specific mutation."

Mutations at the A594V/P/T location occur in about 26.5 percent of the resistant samples, or about 4.5 percent of all samples tested, he said. The M460I/V location occurs in about 20 percent of resistance samples, or about 3.2 percent of all samples tested. Less common mutations are found at the C603W (16.2 percent of samples showing resistance), C592G (5.9 percent), and H520Q (4.4 percent) locations. "We do observe samples that have multiple resistance-associated mutations in the same sample," Dr. Binnicker said. "That occurs about 22 percent of the time among those who have resistance; we see at least two or more specific mutations." The most they've seen is four resistance-associated mutations in the same sample.

Less common mutations occur in the *UL54* gene, he said. The K513N mutation occurs in about 4.4 percent of the samples showing resistance, or less than one percent of all samples tested. "We see some samples that have *UL54* mutations present in them but only about one percent of the total samples tested."

Returning to the case study, the 67-year-old kidney transplant recipient who presented with fever and diarrhea and had initially responded to ganciclovir had a viral load of 280,000 IU/mL 11 or 12 weeks after transplantation.

Using the NGS method, "we detected two mutations in *UL97* [L595S and L595F] that conferred ganciclovir resistance, so the patient was switched to foscarnet," Dr. Binnicker said.

One month later, the patient's viral load increased to 1.8 million IU/mL. The prevalence of the two mutations seen a month earlier had now increased dramatically. "But we saw a new mutation show up—the E756N mutation—right at the threshold [15 percent] for the test that confers resistance to foscarnet, which would explain the increasing viral load," Dr. Binnicker said.

Dr. Binnicker's team plans to add *UL56* to its NGS assay to determine possible letermovir resistance and to study whether routine use of NGS leads to improved outcomes when compared with Sanger sequencing. His team will also explore whether baseline sequencing should be performed in all transplant cases before therapy is initiated, and whether NGS can be used to identify new resistance-associated mutations.

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