Sequencing goes deep to find rubella in uveitis patient

William Check, PhD

November 2016—Metagenomic deep sequencing, or MDS, has scored another coup in the diagnosis of an unexplained disease in a patient who had already had extensive workup with all other available tools. MDS had been used in 2014 to detect unsuspected leptospirosis in a critically ill encephalitis patient, enabling appropriate treatment and full recovery (Wilson MR, et al. *N Engl J Med.* 2014; 370[25]:2408–2417). Now some of the same clinical scientists at the University of California San Francisco Medical Center who helped diagnose that patient have identified rubella virus infection in the eyes of a patient with bilateral chronic intraocular uveitis that had been misdiagnosed as idiopathic inflammation for 16 years (Doan T, et al. *Genome Med.* 2016;8[1]:90).

More broadly, the UCSF clinical investigators reported "a case series of patients in which MDS was used for the first time to identify a range of infectious agents in intraocular fluid from patients with uveitis or intraocular inflammation," neurologist Michael Wilson, MD, MAS, who worked on the encephalitis and uveitis cases, tells CAP TODAY. In addition to rubella virus, organisms included *Toxoplasma gondii*, herpes simplex virus type 1, and *Cryptococcus neoformans*. In two cases of noninfectious inflammatory ocular conditions, metagenomic deep sequencing ruled out infections.

"To our knowledge, this is the first ever report of MDS being used for infectious disease diagnostics on intraocular fluid, which is a very challenging specimen type to work with, given the small volumes that are typically available," Dr. Wilson says. Having a laboratory method to identify infectious causes of uveitis is important, he notes, because of the frequent co-occurrence of eye inflammation with many systemic diseases.

Dr. Doan

Uveitis is difficult to diagnose clinically, says Thuy Doan, MD, PhD, a specialist in uveitis and an assistant professor at the UCSF Proctor Foundation for Research in Ophthalmology, who saw the rubella patient and initiated the use of MDS in the diagnosis. "It will be completely a game changer for ophthalmology to be able to offer MDS instead of a panel of assays for infectious agents," Dr. Doan says. In addition to being limited to very few pathogens, a PCR panel requires much greater volume than MDS, which can be done on 20 to 30 µL of intraocular fluid. "The entire volume of eye fluid is about 3 mL," Dr. Doan says. Only about 300 µL can be safely removed in the clinic.

The MDS method used was developed in the laboratory of Joseph DeRisi, PhD, professor and chair of biochemistry and biophysics at UCSF. MDS on the uveitis patient was performed in his laboratory. "It is absolutely clear to us where we stand with metagenomic next-generation sequencing done in a completely unbiased fashion," Dr. DeRisi says. "I foresee that this technology will likely replace or overtake many of the traditional sendout assays for individual agents.

"Because this technology operates without bias," Dr. DeRisi adds, "it is likely that many more either obscure or unanticipated infectious agents will be captured in a wide range of infectious disease indications."

Making advances toward this goal is possible because of the philanthropic support from the Sandler, William K. Bowes Jr., and Charles and Helen Schwab foundations, which support the UCSF Center for Next-Gen Precision Medicine Diagnostics. Dr. DeRisi is the principal investigator. A grant of $600 million from the Chan Zuckerberg Initiative to establish a Biohub facility at UCSF, which Dr. DeRisi and Stephen Quake, DPhil, of Stanford University

will co-direct, will also greatly aid these efforts. One major project in the Biohub will focus on infectious diseases, including new ways to detect and identify infectious agents (www.bit.ly/ucsf-biohub).

As Dr. Doan recalls it, her first encounter with the uveitis patient was “almost like a CSI case.

“In 2015, when I first started as new faculty here, he came to my uveitis clinic,” says Dr. Doan, director of the Ralph and Sophie Heintz laboratory at the Proctor Foundation. The man moved to Southern California from Germany in 2006. In Germany, he was diagnosed in 1999 with ocular inflammation of one eye that was treated with topical steroid drops. Two years later he had inflammation of the other eye that was treated similarly.

At an institution in Southern California, where he was seen initially, he was exhaustively worked up for an infectious etiology, but all test results were negative. “They thought his symptoms were autoimmune related,” Dr. Doan says, “so he was put on methotrexate and high-dose prednisone for a year.” This regimen didn’t help.
In 2012 the man moved to San Francisco and was examined at UCSF, where the clinicians obtained intraocular fluid for testing. Fuchs uveitis syndrome is known to be due to rubella, Dr. Doan notes. However, the majority of the patients with Fuchs present with inflammation in the front of the eye and in only one eye. “For this patient, the presentation was different,” she explains. “He had inflammation in the front and middle of the eye—both aqueous and vitreous compartments—and in both eyes.” Nonetheless, a viral cause was sought given his characteristic clinical findings (**Fig. 1**).

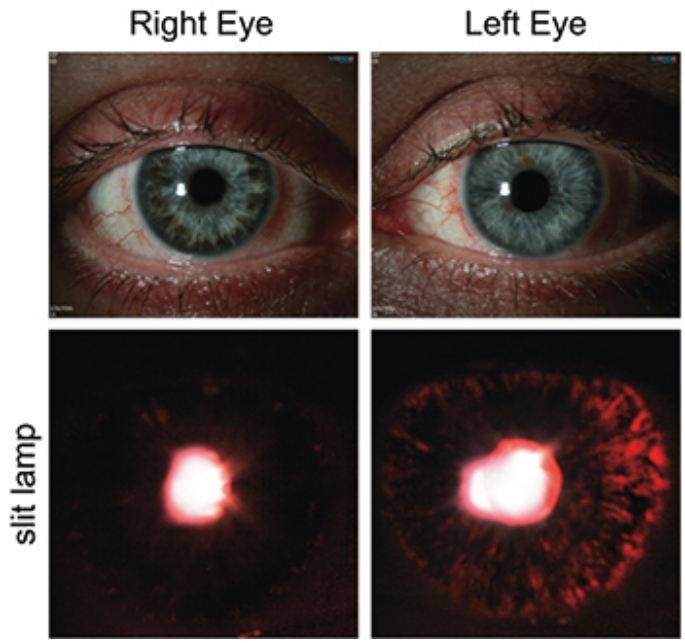

Fig. 1. Ocular findings of a 40-year-old man with bilateral, idiopathic chronic anterior and intermediate uveitis. The top panels show different colored irises (heterochromia) between the right and left eyes and transillumination defects that are prominent in the left eye because of iris atrophy (lower panels). These findings are suggestive of viral-related uveitis. (From Doan T, et al. *Genome Med.* 2016;8[1]:90. Original publisher: BioMed Central.)

At one point, the patient had a diagnostic and therapeutic vitrectomy—removal of all vitreous fluid—and the fluid was sent for testing. “In ophthalmology, we are very elementary in microbiology diagnosis compared to neurology and other fields,” Dr. Doan says. “Here at the Proctor Foundation we have one of the

biggest panels available in the U.S., and we only do PCR for four organisms—herpes simplex virus, *Toxoplasma*, varicella-zoster virus, and cytomegalovirus—as well as bacterial and fungal cultures." Again, all tests were negative.

"Without an infectious cause, I was about to go to immunosuppression again," Dr. Doan says.

She had one last recourse. She knew about Dr. DeRisi and Dr. Wilson's work with MDS in the encephalitis case. So she sought their help in using MDS on the uveitis patient. "We were able to adapt the technology to analyze the patient's aqueous fluid," Dr. Doan says. When she saw the result, she says, "I almost fell off my chair. It clearly showed rubella virus. There was no doubt that it was the organism." Later, the patient reported he had a full body rash and fever that resolved quickly when he was 17 years old.

The sequencing was done in Dr. DeRisi's research laboratory. To verify the result in a CLIA-certified facility for clinical diagnostic purposes, they had the California Department of Public Health do PCR for rubella, which validated the MDS finding. Rubella was confirmed also at the Centers for Disease Control and Prevention.
"In neurology and ophthalmology pretty much the first thing to do is to determine whether the patient has an infectious or autoimmune etiology," Dr. Doan says. "If you make a mistake, the patient can potentially die. This technology is perfect for that situation." It can not only give a positive diagnosis but also rule out infection. "If we can't find a pathogen [with MDS], it's probably not there," she says. "Then you can feel comfortable to give the patient steroids."

On the other hand, Dr. Doan calls giving steroids in the face of an undiagnosed infection "a futile treatment.

"It makes inflammation worse. Now I know how to manage this patient. It doesn't have to be anti-inflammatory drugs or immunosuppression."

Unfortunately, there is no specific antiviral agent for rubella, as there is for herpes. Supportive treatment would be to control high eye pressure and, most important, to do no harm. The latter principle is not simply anodyne. "This patient was on high-dose prednisone for many months and now has osteoporosis as a result," Dr. Doan says. Perhaps future patients with uveitis from an infectious agent will not have to be subject to futile therapy.

Germany does not have a policy of administering rubella vaccine on a population basis, which raised the possibility that the man had been infected in a natural outbreak and that his symptoms in 1993 were a sign of primary infection. As it happened, there was a rubella outbreak in 1992 in the Stuttgart area, a few hours south of where the man lived. The team obtained a sample of the outbreak strain and compared the rubella strain from the uveitis patient to the outbreak strain and several dozen others. Sequencing of the 739-nucleotide sequence of the *E1* gene recommended by the World Health Organization for rubella virus genotyping showed that the patient's strain was a nearly perfect match to the strain isolated from the 1992 outbreak. It did not match any of the others, Dr. Doan says, including the strain used in the U.S. rubella vaccine, which the man received in 2006.

Dr. Wilson

To understand the situation even further, the investigators compared the almost full-length sequence of the patient's strain to that of the outbreak strain nucleotide by nucleotide. Says Dr. Wilson, "We showed that the number, type, and distribution of mutations present in the patient's rubella virus strain [relative to the outbreak strain] were consistent with persistent viral replication under immunological pressure."

To evaluate the implications of the number of mutations, they used data from a study of person-to-person rubella transmission that established the virus' mutation rate. In the patient's strain the number of mutations was characteristic of a 20-year divergence between the two strains, which fits well with the patient's clinical history.
Looking at the location of mutations, they found approximately the same frequency in genes for replication as in genes for coat proteins. However, when they categorized variants as either synonymous (those that would give a protein that was functionally similar to the wild type) or non-synonymous (those that would be expected to alter function), they found a large discrepancy. Non-synonymous mutations were clustered overwhelmingly in genes for coat proteins. In fact, the rate of non-synonymous mutations in coat proteins was about six times that in genes for replication enzymes.

Taken together, these findings suggest that full-length virus had been replicating in the man's eye under immunological pressure since his initial infection in 1993. Because the eye is an immunologically privileged site, the virus had been able to persist as an intraocular infection but unable to spread outside the eye.

Assigning pathogenic significance to a finding from MDS requires skill, experience, and sophistication about sequencing. Often sequences will be found from more than one organism, as happened with four of the patients in this case series, particularly the uveitis patient, whose sample yielded three nonpathogenic bacteria and a handful of background organisms in addition to rubella virus. Most contaminants will be from environmental and laboratory sources, which have to be accounted for in the bioinformatics process.

"For the most part, interpretation of NGS [next-generation sequencing] for viruses is relatively straightforward. We rarely see viral contamination," Dr. Wilson explains. "For bacteria, on the other hand, it is very common, especially when you are analyzing CSF. We see it all the time." Nonpathogenic organisms are picked up as the needle is pushed through the skin. "Interpretation still requires some thought along with clinical correlation and confirmatory testing," he says.

For these reasons, obtaining a final result that unequivocally identifies rubella or any other organism as a causative agent requires a pipeline that includes numerous filters. "Bioinformatics for metagenomic sequencing is becoming increasingly fast and well developed," Dr. DeRisi says. Raw sequencing results are first processed by removing host sequences, which are typically the vast majority of the sample. "Whatever remains is compared to a global database of known sequences, such as NCBI's [National Center for Biotechnology Information] GenBank," Dr. DeRisi says. "We then taxonomically classify those sequences to establish which organisms are present in the sample."

When performing metagenomics it is important to appreciate that virtually any reagent, including water, contains small amounts of nucleic acid, Dr. DeRisi says, cautioning, "Those background environmental sequences have the potential to be confounding." In analyzing the uveitis case, he says, "We take into account the abundance in the sample of the agents detected as well as measuring background sequences present in our reagents, such as water. In each case, for this series of samples, a pathogen was clearly identified" after adjusting for confounders.

Discovering 20 years of a virus replicating in a niche like the eye has consequences for vaccination programs and public health policy, Dr. Wilson says. "It is an unexpected result to discover a long-term reservoir of rubella virus, which was thought to be eradicated in North America. Yet there are these hidden reservoirs walking among us."

Dr. Wilson compares the rubella uveitis incident to the recent example of Ebola virus—like rubella, an RNA virus—found to be replicating in the eye of a person after his body had cleared it (Varkey JB, et al. *N Engl J Med.* 2015;372[25]:2423–2427).

Dr. Doan raised the possibility that this may be happening also with Zika virus. "The eye may be the perfect site for Zika replication," she says. More generally, "The eye may be a reservoir for pathogens." However, virus in the eye may not be easily transmissible, she says: "In the uveitis patient, there was no virus being shed in tears. It was confined to the intraocular compartments."

Thinking more broadly, Dr. DeRisi speculates, "The ability to comprehensively and sensitively assess ocular

infections with a single assay could have far-reaching implications for public health surveillance regimes that monitor emerging and re-emerging infections."

Dr. DeRisi

After the encephalitis case, metagenomic deep sequencing on cerebrospinal fluid at UCSF was moved into a CLIA-certified laboratory, an effort led by Charles Chiu, MD, PhD, associate professor of laboratory medicine and medicine/infectious diseases at UCSF. He and Steve Miller, MD, PhD, director of the UCSF Medical Center microbiology laboratory, also played major roles in using MDS to diagnose the encephalitis patient. Says Dr. DeRisi, "We develop the methods and technology in the context of research studies, then advance it into a clinical lab so it becomes broadly available to patients." In the future, Dr. Wilson adds, "We want to move MDS on other bodily fluids into a CLIA-certified lab as well, such as ocular fluid and bronchoalveolar lavage for pneumonia."

The California Initiative to Advance Precision Medicine is sponsoring the work to advance the movement of MDS out of the research laboratory and into the clinical laboratory. It is funding two collaborative demonstration projects, one of which is the Precision Diagnosis of Acute Infectious Diseases project, conducted at three University of California sites (www.bit.ly/ciapm-project). Dr. Chiu is the principal investigator for the project.

William Check is a writer in Ft. Lauderdale, Fla.