Advanced Cell Diagnostics

Insights into viral pathogenesis

Determination of causation by highly sensitive and specific viral RNA detection and localization with RNAscope® ISH

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Understanding the biology of interactions between viruses and their hosts is crucial for creating intervention in dealing with the causative impact of viruses in agriculture industry and human health. Dr. Patricia Pesavento, professor of Pathology, Microbiology & Immunology at UC Davis, spoke to us about how her research in this area was greatly influenced by her decision to adopt and routinely use RNAscope® ISH. With its high sensitivity and spatial resolution, RNAscope is proving to be an essential tool for understanding the causative role of virus in varies diseases and a vital technique in studying the viral pathogenesis.

Can you tell us about your background and work?

My decision to go to veterinary school came from wanting to understand the host reaction to a pathogen. I was introduced to pathology in my second year, and I haven't looked back since. In terms of my current role as a faculty member specializing in anatomic pathology, my work includes teaching, diagnostics, and research. In our diagnostic work, we examine biopsies and necropsies and try to recognize the effect of viral disease in different types of host animals, and the range of submissions to the veterinary school at UC Davis can be anything from elephants to tarantulas!

My research focus is viral diseases, and a large part of my lab's research is done in collaboration with the virus hunter, Professor Eric Delwart, at the Blood Systems Research Institute at UCSF. While the Delwart lab focuses on discovering and identifying the viruses, my group focuses on recognizing the damage done by the virus. Viruses are very clever considering how simple the structure they have. We recently have discovered several polyomaviruses. Their genomic DNA is only 5 kb long encoding only three structural proteins and one oncogenic protein, while the range of tissue reaction to this family of viruses is astounding. Polyomaviruses can be cytolytic, benign, or oncogenic. It's very humbling to study them. Their defense against the animal host is a product of hundreds of thousands of years of evolution.

What's the real-world context of looking at viral-host interactions?

Firstly, studying diseases in wildlife and pet animals is important for its own sake, i.e. for animal health, and herd health, but the bigger picture is ecosystem health and understanding how we as humans are affecting the world around us.
There is also significance for humans, and the detailed context is, to me, very fascinating. The research world has a strong history of studying experimental animal models for understanding disease, but we are now able to recognize that the mouse isn't always comparable to a human and we can't always mimic pathogenesis in these animals. However, humans do share features of susceptibility and reaction with other creatures. Looking more broadly at multiple host reactions

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provides insights into how we are, or potentially could be, affected by the pathogens around and within us.

Even more exciting for me is looking how one type of virus affects different host animals: they often react in a remarkably similar way, but they also exhibit species-specific differences. For example, herpes is capable of jumping species and leading to a devastating outcome. There is a type of herpes, that can jump from sheep into other ruminants, like deer, cows, or buffalo, and while the sheep carry the herpesvirus with no detectable symptoms, once herpes gets into another animal, it becomes a fatal disease.

How do you discover a virus and its contribution to disease?

As an example, I would first look at a case or an outbreak of an illness such as pneumonia or diarrhea. Through examining the diseased tissues, the host symptoms, as well as how it spread, I can identify with a reasonable level of confidence whether a virus is contributing to that outbreak. Once we determine that a virus might be the causative agent, we can use either a degenerate primer strategy if we know a particular viral target, or the metagenomic analysis performed by the Delwart lab to identify the virus within the tissue samples that I provide. In the case of pneumonia, for example, a piece of lung tissue is used.

It is predicted that there are as many as 10^{31} novel viruses as yet to be discovered, though, most of which are completely innocuous. With so many novel viruses out there, employing methods that examine populations, such as NGS for metagenomics, means that Eric will always find one - but this may not necessarily be the causative agent.

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In order to determine if the virus identified was responsible for the symptoms originally observed, we must visualize the virus within that tissue or within that lesion. This is where RNA *in situ* hybridization (RNA ISH) has become such an important tool for us.

How does RNAscope ISH facilitate your research?

Defining viral pathogenesis is challenging because each host responds differently to the same strain of virus, hence it is not very informative by identifying the host symptoms. In addition, approaches such as PCR or metagenomics with NGS can tell you that a virus is present, but they can't tell you if the virus is causative since there are so many viruses in the world and it's not hard to detect one. Therefore, a method as sensitive as the RNAscope assay with spacial resolution is absolutely irreplaceable for our confidence in determining viral contribution to a disease.

RNAscope ISH facilitates our research in two ways. Firstly, it assists us in identifying the target cell or tissue where the virus is located, and secondly, it provides us with insights into viral pathogenesis, including which type of cells is the target for infection, and how it is affecting that target cell. We achieve this through designing RNA probes to identify whether or not the virus is expressing protein coding genes involved in viral division and replication, or to identify important processes such as transformation in the case of oncogenic viruses. From sequence information that we provided, the ACD team have designed custom RNAscope probes for our projects. With its importance in understanding causality and the viral-host relationship, RNAscope technology has been a vital tool in my research and has supported my collaboration with Dr. Delwart.

Can you tell us in more detail how you have applied RNAscope ISH?

We've relied on RNAscope ISH in many studies, leading to some exciting discoveries, particularly in cattle and raccoons.

Neurological disease in cattle

In light of bovine spongiform encelopathy (BSE), the US has a strong surveillance system for neurologic disease in cows. Our diagnostic lab received a sample from a cow with neurologic disease. After testing negative to a host of diagnostics for known causes of neurologic disease, we sent a brain sample to the Delwart

1 - **Divergent astrovirus associated with neurologic disease in cattle.**
Li L., et al. (2013). Emerg. Infect. Dis.;19(9):1385-92.

2 - **Neurotropic astrovirus in cattle with nonsuppurative encephalitis in Europe.**
Bouzalas I.G., et al. (2014). J. Clin. Microbiol.;52(9):3318-24.

3 - **Astrovirus VA1/HMO-C: an increasingly recognized neurotropic pathogen in immunocompromised patients.**
Brown J.R. et al. (2015). Clin Infect Dis.;60(6):881-8.

4 - **Diagnosis of neuroinvasive astrovirus infection in an immunocompromised adult with encephalitis by unbiased next-generation sequencing.**
Naccache S.N., et al. (2015). Clin. Infect. Dis.;60(6):919-23.

5 - **Merkel cell carcinoma: a virus-induced human cancer.**
Chang Y. & Moore P.S. (2012). Annu. Rev. Pathol.;7:123-44.

6 - **Novel polyomavirus associated with brain tumors in free-ranging raccoons, western United States.**
Dela Cruz F.N,.et al. (2013). Emerg. Infect. Dis.;19(1):77-84.

lab to test for the presence of viral species. From this investigation, a completely novel astrovirus was discovered. This was surprising, since in most animal species the astrovirus family is exclusively a pathogen of the intestinal system, and therefore its significance as an underlying cause for encephalitis was not without suspicion.

Working with us, ACD designed a custom RNAscope probe to recognize the astrovirus, and our RNA ISH studies confirmed a strong presence of this virus within degenerating neurons of the cow's brain. From this study, we determined that this bovine astrovirus is a neurotropic form[1,] and in the two years since this study, other papers have been published describing two human neurotropic astrovirus cases[2,3,4].

Subsequent examination of FFPE (formalin fixed, paraffin embedded) samples from cows in Switzerland with neurologic disease of unknown origin also identified the same astrovirus as the causative agent[2]. Our work has really contributed to understanding this population of diseases with an unknown origin. We are beginning to recognize that, although very rare, certain types of astroviruses are able to infect brain tissue. Research in this area is growing, and RNAscope ISH is providing convincing evidence to support this.

Polyomaviruses in raccoons

This project is really fascinating. Although the polyomaviruses have long been known to cause tumors in laboratory animals, the first natural tumor associated with polyomavirus (Merkel cell polyomavirus) was only discovered a few years ago[5].

We've recently identified a population of raccoons with brain tumors that are 100% associated with a novel polyomavirus (RacPyV). Tumors in raccoons are usually rare, but in the past four years, we have found a significant number of cases (12%) with brain tumors which are indeed due to RacPyV infection[6]. Our research aims to understand the causes underlying this outbreak, providing insights, if possible, into the potential pathogenesis of this virus family.

RNAscope ISH has facilitated this project by allowing the analysis of viral gene expression in the tumor, especially where and how the virus lives in the host animal over long periods of time. In this project, we utilize two RNAscope probes: one probe detects the oncogenic gene of the virus (LT antigen), and the other probe detects the viral structural gene for the viral capsid protein. This capsid protein is expressed in particular during viral replication and continuously expressed during shedding for transfer into another host, and therefore, it is an ideal biomarker for identifying the cytolytic stage of the viral lifecycle.

Using these probes, we can identify the persistence sites of viral infection in different tissues and compare different tissue responses to the virus. For example, kidney tubular epithelium is one of the persistence sites which is confirmed by developing primary cell lines from the target tissues, but no tumor is developed there. However, in the brain, the virus expresses a high level of oncogenic gene transcripts but almost none of the structural gene transcripts. This indicates that the virus infected the brain somehow only focused on replicating the viral oncogene, which in turn to drive infected brain cell division and causing tumor development.

The next step in this project is to develop primary tissue cell lines from infected raccoon. By defining the differences between the brain and kidney infection sites, we will be able to reveal how the cellular microenvironment altered the patterns of viral gene expression.

Do you often use FFPE samples with RNAscope ISH?

Yes, here at the UC Davis veterinary school, we have 50 years of cases cataloged in FFPE tissues. Detection of viral presence in just one animal is not nearly as powerful in terms of establishing causation as having a series of animals with similar molecular signatures. By using RNAscope as a reliable detection assay, it helps us to create a standard protocol to define how and when a virus might be contributing to a disease.
Take the case of pneumonia in dog for example:

"Currently, only RHAscope ISH has the sensitivity and specificity I'm looking for."

once a novel virus is discovered, the viral sequence can be used to design probes for RNAscope ISH to detect if this virus is present in lung tissues. Once confirmed, the same probes can be used to screen through the archived samples. This will help us understand whether this newly discovered virus has been contributing to dog respiratory disease over time.

Do you use immunohistochemistry alongside RNAscope ISH?

I do use immunohistochemistry. However, as you know the IHC technique is limited by the sensitivity and specificity of the antibodies in pathologic tissues. These limitations are particularly evident when detecting a tumor-associated papilloma virus in horses. During tumor formation, the virus does not express most of the viral proteins, rendering antibody-based detections of these viral proteins ineffective. Instead, RNAscope ISH probes are specifically designed to detect the viral genome with high sensitivity and specificity. The virus was able to be detected in all the tumor cells in the horse tumors examined, indicating a viral contribution to tumor development. Moreover, using RNAscope technology, we visualized two types of viral-associated tumors in horses: one caused by bovine papilloma virus (sarcoids) and one by equine papilloma virus (genital squamous cell carcinoma). RNAscope ISH is a very good complementary technique to protein-based analysis in epidemiologic studies.

How does RNAscope ISH compare to other RNA ISH methods?

In the past, I have used oligomer probes which were highly specific but lacks sensitivity. As part of the bovine astrovirus project, a side-by-side comparison was performed and we found a significant difference between the two methods. Oligomer probes gave a false negative result for identifying infected cells in brain tissue sections. In contrast, RNAscope ISH enabled us to clearly identify multiple infected neuronal cells in the tissue sections and generate novel data for us.

How do you hope to apply RNAscope ISH in future research?

I'm looking forward to using RNAscope technology for the investigation of splice variants in oncogenic viruses. In the case of polyomavirus, there is only a single gene that is thought to be oncogenic, but there is much debate over which splice variants of that gene are driving the cells towards tumor formation. One of the ways we proposed is to look at different probes targeting different regions of the gene and to demonstrate where and when that gene is expressed during the progression of the tumor. Currently, only RNAscope ISH has the sensitivity and specificity I'm looking for. I am very excited to see what RNAscope can help us further advance our research on viral pathogenesis in the near future!

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