



WINN FELINE FOUNDATION

For the Health and Well-being of All Cats

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36th Annual Winn Symposium – June 26, 2014

“Glimmers of Hope-HCM and FIP”

Transcript of Audio: Introduction and Beth Licitra on FIP

Steve Dale: Well, welcome to the 36th annual Winn Feline Foundation Symposium. My name is Steve Dale and I am on the board of directors for Winn Feline Foundation. I thank you all very much for coming. So, once again, to all of you I say welcome! [applause] That’s what I like to hear! So, if we see those slides of clogged arteries, we should have applause. We want enthusiasm.

I have some housekeeping sorts of things to talk about. Please complete the symposium participation survey and return it, especially veterinarians – and there are veterinarians here – since their surveys are used for future RACE program approval, and veterinarians will know what I am talking about. Veterinarians and staff should remember to get their CE certificates before they leave. Turn your cell phones on vibrate or off. Anyone who would like to make a donation to the Winn Feline Foundation throughout the course of the night, Vickie Fisher, our treasurer, she is the lady who is all about the money, up in front; raise your hand again, Vickie. Okay, we are more than happy always to take your money.

I have a couple of announcements to make that I am going to save until later, but my job is to tell you a little bit about – and I am sure most of you know a great deal about – the Winn Feline Foundation. The symposium, as I said, this is the 36th annual symposium that is being held. Pretty much a who’s who of veterinary medicine and we continue that today with Dr. Fox, a who’s who of veterinary medicine, has participated in the Winn Feline Foundation Symposium over the years. This symposium that you are at that has had that who’s who of veterinarians who appear, these symposiums that encourage further research would not have been possible if it wasn’t for a lady sitting in the back of the room, whom I am about to introduce, the great Joan Miller. [applause] She deserves that applause and much more for what she has done for cats for all these years. Occasionally I have had the opportunity to work with Joan; a learning experience every time I do. Someone here said she is a smart lady. Whoever said that is very right.

The Winn Foundation is a not-for-profit organization that goes back to 1968. If any of you have cats in the room ... By any chance, does anyone here have a cat? [laughter]

Woman: I have one.

Steve Dale: Only one?

Woman: A big one.

Steve Dale: You might have the fewest!

Woman: No, no!



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Steve Dale: The Winn Feline Foundation is responsible so much medically for everything about that cat, in fact, more than just medically. Everything from what our cats eat on a day-to-day basis, to the shelter cats we might adopt, to the way in which they are kept in shelters, to the medical care that they receive throughout a lifetime, from vaccines to preventive care, to a whole lot that goes into senior care, to treating diseases like two that we will hear about today. The Winn Foundation, over the years, has had everything to do with funding for that. Here is sort of a best of the best list; I am not going to go through all of these, but the idea is, for any of you who talks to ... because I am sure most of you ... I'm preaching to the choir here, right? You know about the Winn Feline Foundation, but here is how you can help us. Each and every one of you can be an ambassador to spread the word that all of this stuff and more, having to do with cats, the Winn Foundation, if it was not for us funding the researchers doing the work, well, these things might not have happened or would have happened much later.

So, we are going to hear about two topics that mean a great deal to me personally and a great deal to your average cat owner. You guys are not the average cat owner, but my guess is these topics mean a lot to you too. We don't know how many cats die of heart disease or not. By the way, anybody know this cat? Or about this cat? Yeah. Do you remember this cat at all? Anybody? So, back in the day ... and I will tell the brief story. I had a dog that did animal-assisted therapy and my wife said to me ... That is when dogs go to hospitals or nursing homes or rehab facilities to help people feel better and, in some cases, help people to get better. My wife came back one day and said, "You know, we have to teach our dog ... " (a miniature Australian shepherd named Lucy) " ... we have to teach her something new." And I don't know why, but I bought a little miniature piano, that was it, and I began the process of clicker training, if any of you know what that is about. It is operant conditioning. You click the clicker and the cat raises its paw just a little bit toward the piano and you click it again, give a treat, and you get closer and closer; in fact, Lucy was lifting her paw a little bit. We were beginning to shape the behavior. It takes a little while to do that. You cannot become a prodigy overnight, necessarily. I had closed the door so the other pets would not come into the room, but I did not close it all the way, and in walks that little Devon Rex cat that looks at me, looks at the dog, looks at the piano and just goes ping, ping, ping, ping, ping, ping, ping, ping and I thought, "Well, what am I fooling around with this dog for? I've got a musical genius here."

So, Ricky was already a very social cat and what this did ... and thank goodness for the breeder. Of course, we had the breed going for us; the Devon rex happen to have a certain personality that kind of lend them that way. She was socialized when she was young. That's what I mean thank goodness for Leslie Spiller, the breeder of this cat. I was able to take this cat out into the world and Ricky loved it. As a result, we had a bond that I doubt I will ever have with another cat and maybe not even a dog. We did everything together. Before all was said and done, Ricky knew more than just to play the piano.

Any of you have little kids or know any? So, what you could do is line them up one by one by one by one by one by one by one and Ricky would go hop, hop, hop, hop over each one of them. He would jump over them, you know, kind of his own version of feline agility. He would sit and give a high four, not quite a five. I would go through a whole litany of things. I would make up stuff or Ricky would make up stuff. If I wanted Ricky to jump from this chair to this chair, like where you are sitting to this chair over here where you are sitting, I could just, at some point, point and Ricky had never done it before. We knew each other that well. Ricky enjoyed performing. Loved cameras, whenever cameras came around as TV crews did. Then, one day, we went to the veterinarian and my veterinarian's face just turned white as she was listening to our cat's heart. Before she said anything, I kind of knew and we went to see the cardiologist. Ricky lived for several more years without any symptoms, began to

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have a few, and that is when I said, “This is ridiculous. We have to do something about it.” And we did. We began the Ricky Fund.

The Ricky Fund has raised ... Why did we? Because there is no cure, there is no really effective – or wasn’t then – really effective treatment. We are going to hear if there is a really effective treatment in a little while for HCM, but it was frustrating and what I didn’t know then is how many cats actually get this. And it is not only pedigreed cats; it is any cat at all, potentially. So, we are going to hear about that. We began the Ricky Fund. It has raised, to this minute, well over \$100,000, so I thank you all very much for that. Really, thank you very much for that. As a result of that, as Maine Coon and ragdoll folks know, there is a simple blood test that can be done to determine if the gene defect is there. It is not a perfect test, but what is nice about it is we have been able to save some lives and that is awfully nice, but I want to do more than that.

We can skip past that. Then, a couple of years later, we heard from Susan Gingrich – yes, Newt’s sister – and she said, “I had this lovely sweet cat named Bria.” And, as often happens with feline infectious peritonitis, it is a kitten, and the cat owner has no idea that this disease even exists. Why do people – I am not talking about all of you, but in general – why do cat owners typically get a kitten? Maybe it is because another cat had passed away, right? So, they are grieving that cat that had passed away. They get this little kitten. They go to the veterinarian, who says, “Oh, by the way, this kitten has a disease that you’ve never heard about. Your kitten’s going to die.” It is the most devastating thing. I would argue for veterinarians, it is the most devastating news that a veterinarian can offer a client and the most frustrating thing for a veterinarian to deal with. Cancer, often occurs in older cats. Lots of kinds of cancers can be treated. FIP, that is a tough one and it is something ... The good news is, there are so many people who are so passionate about it and, in fact, we have raised, similarly, more than six figures in the Bria Fund.

Dr. Al Legendre spoke – it was two years ago – at the Winn Feline Symposium. He offered some really good news. Niels Pedersen has offered some good news. In fact, the Winn Feline Foundation, with Niels Pedersen and Niels Pedersen independently, are responsible, I would say, for almost everything we know about FIP, but now Gary Whittaker is on the scene as well at Cornell and, in fact, we are about to hear all about it as I introduce Beth Licitra, a current combined DVM/PhD student but about to be a veterinarian in a matter of days, which is very, very cool. Isn’t that nice? [applause] I had the chance to talk with her earlier today and wow! What a smart, smart lady who is also committed to do something about FIP. Her research in the Whittaker Laboratory focuses on investigations into the initial steps of the virus infection. This includes binding of the virus to its host receptor, activation of viral attachment proteins by host proteases and fusion of viral and host cell membranes. Please help me welcome soon-to-be-doctor Beth Licitra.

Beth Licitra: Thank you very much, Steve. It is a real privilege to be here today. I attended the Winn Symposium two years ago when it was held in Boston and I heard Dr. Leslie Lyons speak about her work on feline genetics and I have to say, it is a little surreal to be back today to speak to you all about my work on FIP. I want to lead off by acknowledging the organizations that have made our work possible. Winn has been a great supporter of our research over the years. We have also received funding through the Morris Animal Foundation, through the Cornell Feline Health Center, from ANTECH Diagnostics, and my funding as a combined DVM and PhD student comes from the veterinary college through private endowments.

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We have had some excellent collaborators over the years. Dr. Gerald Duhamel is a pathologist at Cornell and he was really instrumental in helping us access immunohistochemistry-confirmed cases of FIP from our pathology archives. Dr. Kornreich, who is over at the Cornell Feline Health Center, has been helpful in connecting us with cat owners who call in with questions about FIP. Scott Moroff was a veterinarian at Angell Memorial Hospital for most of his career and now currently he is the vice president and chief scientific officer at ANTECH Diagnostics and I cannot say enough nice things about Scott. He has been really supportive of our idea to take the mutation that we have identified in FIP and use it as a basis for a test to discriminate between feline infectious peritonitis virus and feline enteric coronavirus. He has really given me some great opportunities. I was able to present ANTECH's work last year at ACVIM and that is how I met Dr. Thayer and I really could not be more grateful to Scott for that. And there is Ed Dubovi, who is a virologist in our diagnostic lab, who has contributed to our work and a special thanks to all the cat owners, veterinarians and shelter personnel who have contributed to our work over the years.

I have to say on a personal note, I think for us in the lab it has been really eye-opening to be able to interact with cat owners in this way because generally in the lab, you do not have that client interaction that veterinarians have and you do not see the clinical side of FIP. So, having the owners call us very desperately looking for a clinical trial, a potential therapeutic, has really affected the way we approach to this problem. Yes, as virologists, we are really interested in understanding how enteric coronavirus is able to infect macrophages and cause FIP, but we are also interested in development of better diagnostic tests, in testing out new therapeutics and, ideally, one day designing a better vaccine.

I have divided this talk into six parts. I am going to open with a little bit of fun history about FIP research at Cornell, then I am going to talk about coronaviruses in general and introduce the idea of the internal mutation hypothesis. In the third section I am going to delve a little bit into some biochemistry, into some virology, and I really hope that I can communicate the science in a way that helps you to better understand our research. In the fourth section I am going to talk all about the work that was published last year in emerging infectious diseases and what that means for potential therapeutics for FIP, and then we are going to move into what is the ultimate goal for all of us in this room and that is a treatment for FIP. I am going to end on a really clinical note. I am going to talk about the diagnostic tests that are currently available and I am going to present some data that has come out of ANTECH on the tests that we are working on together and give you my perspective of how I think that test may be used in the future.

So, if you have never been to Ithaca, please come visit us. It is beautiful. You can see Cornell is a lovely campus. Try not to come between December and May because that is winter. In the summer it is really stunning.

We are all really proud of Dr. Jean Holzworth. She was the only woman to graduate in her class in 1950 and she went on to be one of the pioneers of veterinarians and feline medicine. In 1962 she was the first person to describe FIP and I want to read to you her description. This is the very first publication of FIP in the literature:

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“Some Important Disorders of Cats.

A peculiar entity with a definite predilection for cats is chronic fibrinous peritonitis, in which fibrin deposited on the abdominal organs, especially the liver and spleen, gradually organizes into a tough, pale fibrous coating. The liver and spleen may become contracted into barely recognizable forms. Clinical signs are persistent fever, gradual loss of weight and appetite, and enlarging of the abdomen with a more or less clear fluid. The condition is seen most often but not invariably in kittens and young cats, often in several in a household or cattery. Respiratory infections and lavish dosings of various antibiotics appear in many of the histories. To date no causative organism has been isolated or any effective treatment found.”

This is a really classic definition of effusive or wet FIP and if you fast forward 20 years to the laboratory of Dr. Fred Scott, at this time he knew that FIP was caused by infection with a feline coronavirus. Fred Scott did some really pioneering work to characterize antibody-dependent enhancement. For those of you who are not familiar with it, that is a phenomenon that occurs when some viruses infect macrophages in which antibodies against that virus actually enhance infection of those cells. This is what happens in FIP, it happens in dengue hemorrhagic fever, and the virus is able to coat itself in host antibody and then use those antibodies like a Trojan horse to enter through an Fc receptor, a specialized receptor on the macrophage, like coming in through the back door. So, that has really presented a problem for us in designing an FIP vaccine because the point of a vaccine is to stimulate an immune response, but if you stimulate a strong antibody response, you are going to predispose that animal to developing fulminant FIP.

Dr. Scott also studied FIP’s predilection for macrophages and I have to say that a lot of the work we are doing today in Gary’s lab is just a continuation of things that were started by Dr. Scott.

Back in 1974, he founded the Cornell Feline Health Center. This year they celebrated their 40th anniversary and I wanted to highlight some of their recent achievements. They have recently expanded their grant program so they are able to address more problems in feline medicine and that is really terrific.

I want to introduce to you Dr. Gary Whittaker. Here he is in the back row there. Gary is a biochemist by training. He did his graduate work in the United Kingdom. He came to the United States to Yale for his postdoc where he worked on influenza and he spent probably the past 25 years researching influenza. He really recently began to work with coronaviruses. He studied SARS coronavirus when it emerged in 2003 and recently in the lab we have been working with MERS coronavirus, if any of you have heard of Middle Eastern respiratory syndrome. We actually have that virus in a VSL3 facility and one of our postdocs has been doing some research on that. As an interesting side note, MERS is actually capable of infecting human macrophages, so that is something that one of our postdocs is looking at and it certainly correlates with FIP. What is great about working for Gary is that he approaches this veterinary problem from a new perspective. Since he has integrated what we know about flu, which is a much better studied infection than feline corona and taken that knowledge and applied it to feline coronavirus and I think that is really what allowed us to make the recent breakthrough that we did in research that I will talk about tonight. I also want to point out this man here, Jean Millet, who is a postdoc in our lab. He is the postdoc who is currently working on MERS and he helped me with some of the work in the study. He is coauthor with me on our paper that was in Emerging Infectious Diseases.

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Part Two: The internal mutation hypothesis. I want to create for you a paradigm of coronavirus infections as they occur across species, not just the cat. These are endemic pathogens, so animals are exposed and develop some level of immunity at a really young age. MERS and SARS are not examples of classic coronavirus infection. These are examples of recent post-transmission events from other species. They are quite virulent. You may not be aware that in people most coronaviruses cause the common cold; 20% of common colds are caused by a coronavirus. These pathogens, they have an affinity for the respiratory and enteric tracts and there they cause really mild self-limiting infections. You really only see severe disease in immunocompromised animals, so animals that are young, whose maternal immunity is waning, and who have not fully developed their own immune systems. Animals that are under stress like high-density housing situations. In the case of bovine coronavirus, very cold winter conditions for cows or for postpartum cows we can break with bovine coronavirus. In cats, we know immunocompromised FeLV is also a trigger for feline corona.

Coronaviruses are named for the distinctive proteins that project from their surface. The word corona is Latin for crown and these projections right here on the viral envelope are known as spike proteins. I want to introduce spike early in the talk because it is really the focus of our research. Spike is critical for determining which cells in the body will be infected. That is because it binds the host cell receptor and it also initiates fusion of the viral envelope here with the host cell membrane and that fusion event culminates in release of the viral genome into the cell and subsequent infection.

Coronaviruses are really fascinating from an evolutionary standpoint because they are capable of this rapid evolution to infect new cell types and new species. There are multiple reasons why coronaviruses are able to adapt so quickly. I am going to go through each in some level of detail.

First, coronaviruses can swap genes to form recombinant viruses. Second, they have these huge RNA genomes and these RNA viruses, as a general rule, unlike DNA viruses or unlike eukaryotic organisms, have a really error-prone copying mechanism. So, we know that the coronaviruses, feline coronavirus has a very big genome, it is estimated that for every genome that is copied, you have three mistakes, so there is a huge potential for variation in coronaviruses and for selection of mutants that are better adapted to the host, and then coronaviruses are able to use multiple host cell receptors and the type of receptor that is on that tissue type or that cell type helps to dictate whether the virus can infect that cell.

I want to lead off by talking about the propensity of coronaviruses to form recombinant or chimeric viruses. In the rare instance that you have a cat that is coinfecting with a feline coronavirus and a canine coronavirus – and this can happen because both viruses are really highly related and they use the same host cell receptor – you can have the production of a canine corona/feline corona recombinant and I must say this probably happens very rarely, but it has happened, and then once that variant is formed, it can start circulating in cats.

It turns out that in cats today, there are two genotypes of feline coronavirus circulating and this type, coronavirus type 2, is a recombinant with canine corona. It actually has a canine coronavirus-like spike protein and it makes up about 10% of cases of coronavirus infection in cats. It is really easy to grow in a laboratory setting, so all of that work that I was talking to you about that Fred Scott had done has been done on type 2 coronavirus. The problem is that is not the clinically relevant form of the virus. Clinically, 90% of cases are caused by a type 1 coronavirus and this virus does not grow in the laboratory. We believe that has something to do with the type of

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receptor this virus needs to enter a cell. It is not a receptor that you can find on cells that grow in culture. It is a receptor we believe is on only primary cells in the organism. It is the focus of our research a) because it is clinically most important and b) because we do not know as much about it. Things get a little confusing when it comes to coronaviruses and the disease they cause in cats. Whether your cat is infected with a serotype 1 coronavirus or a serotype 2 coronavirus, the outcome can be on a very big spectrum. You can either have feline enteric corona, which is ubiquitous coronavirus that is in the environment that most cats and multi-cat households are exposed to. It is limited to the GI tract. These cats are able to clear the infection and are healthy. You can also see the development as feline infectious peritonitis and this form, as you all know, has systemic consequences; it is pretty much universally fatal.

FIP, to complicate things a little bit more, can present in either a wet or a dry form, so you can have effusion, the wet form, or non-effusive disease, the dry form. It is believed that what form of FIP the cat presents with has to do with that cat's individual immune response to the virus. To get back to some neat things about coronaviruses that allow them to evolve. Coronaviruses have accessory genes. Why is this important for FIP? Well, it turns out some of the mutations that have been identified by Niels Pedersen that are correlated of FIP are immune accessory genes. We do not really know what these genes do. They are not required for replication and cell culture, but they do seem to have a role in infection and pathogenicity in vivo so that they may modulate the immune response to enhance pathogenicity in that way. Niels Pedersen, you probably are all familiar with the mutation in 3c that correlates with FIP, he has also reported on a mutation in 7b. So, these accessory genes are really unique to coronaviruses and I wanted to introduce that.

Coronaviruses, as I have already told you, have a really high mutation rate, so when your cat is infected with a coronavirus, you do not want to think of it as having a single virus. You want to just think of it as being infected with a quasispecies of viruses. What do I mean by quasispecies? A quasispecies is a group of highly related but slightly different viruses that are competing within the same environment. Like I talked about, every viral genome could have three mutations from a parent genome. I tried to make it so in this little quasispecies cloud here, all the viruses are blue and different shades of blue. These are all really closely related viruses, but they are all a little bit different. You can see in an animal that is immunosuppressed, that is not controlling replication of the coronavirus in the gut, you can have the selection of a mutant ovarian that is able to cause FIP.

I am going to gloss over this a little, but I want to highlight that another thing that is important for coronaviruses in general is their ability to use different receptors and one idea we have in our laboratory is that feline enteric corona might enter enterocytes of the small intestine by using a different receptor than the receptor that it might use when it enters a macrophage. I have already told you in antibody-dependent enhancement there is this Fc receptor, the Trojan horse, that coronaviruses can use, but they can also use a molecule called DC-SIGN. DC-SIGN is best studied in relationship to HIV. It turns out that this is expressed on dendritic cells, which are an immune cell as well as macrophages and other immune cells. HIV is able to latch on to this DC-SIGN receptor and get carried to the lymph nodes where it can infect its target cells, which are T cells in the lymph nodes, and it appears ... Work in our lab has shown that feline coronavirus can also use DC-SIGN to enter host cells and this was done by a postdoc in our lab, Andrew Regan, a few years back. So, it is just to say that when we think about the transition of enteric coronavirus to FIP, there are multiple factors that could be at play. I think that is something that is really coming out now. There is probably not just one mutation that causes FIP. It might be a

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constellation of changes in the virus and that might explain why it has been so hard to pin down the genetic change in the virus that is linked to the development of FIP.

Okay, so this brings us to the internal mutation hypothesis and I tried to convey the point that the hallmark of FIP is its ability to productively infect and activate in macrophages. It is thought that FIP arises uniquely within each infected animal from an enteric coronavirus. So, if every animal has a virus that experiences a mutation that causes FIP, we do not believe that FIP is transmissible. We believe that is what is transmissible between cats – this is what research shows – is the enteric form of the coronavirus and it is that virus then can lead to FIP based on how the cat responds and controls that infection.

Okay, so I have a little animation here. Feline enteric coronavirus is transmitted by the fecal-oral route, so cat ingests the virus, goes to the small intestine where it begins to replicate. It is excreted in the feces and for most cats, that's it. Their immune system controls the infection and they clear the virus. Some cats – this is work that has come out of Niels Pedersen's laboratory – become persistent shedders. The virus persists in their intestinal tract and they continue to shed coronavirus into the environment and these are the cats that in your catteries or in your shelters are consistently reinfesting your queens or your new litters of kittens and they themselves may not end up breaking with coronavirus. However, some cats that cannot clear the infection, young cats, cats with FeLV that are otherwise immunosuppressed, in those cases a mutated variant of the virus can arise that spreads in macrophages and triggers FIP.

An alternative theory to the origin of FIP is the circulating strain hypothesis. In this hypothesis, both pathogenic and nonpathogenic variants are actively circulating among cats. Whether a cat develops FIP is determined by what strain they are exposed to. I have to say that in Gary's lab, our current thinking really takes these two hypotheses and combines them. They are not mutually exclusive. So, while we think that FECV is transmissible, a mutation does occur within an individual animal to lead to FIP, not all FECVs are the same. Some FECVs in circulation probably carry a number of mutations that make them predisposed or closer to becoming FIP and that could explain why, in certain situations, in certain catteries, in certain shelters, you see what appear to be outbreaks of FIP because those cats are infected with an enteric corona that is very likely to mutate to FIP. That is something that we have not tested, but something that we are looking to test by looking at samples that have come from cats in environments where an outbreak is ongoing.

Okay, this is where we get into some biochemistry and a little bit of virology. All the viruses on this slide have one thing in common and that is that in order to infect their target cell, they need to be activated by a host enzyme or protease and I am going to break this down step by step. So, spike, which we have talked about, is the critical determinant in which cells in the body will be infected and that is because a) it is responsible for binding to the cell receptor and b) it triggers that fusion event where the viral envelope and host membrane fuse and the genome gets released into the cell. None of this can happen until the spike protein is activated by a host cell enzyme. This is a common theme among some viruses, that this is how they control infectivity, and if you look at the coronavirus spike – I have given you a linear depiction of it here – coronaviruses have two regions in the spike protein where they can be acted upon by a host protease to be activated.

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I think I was in the lab about a year and a half before I really understood what a protease was because everyone in my lab has a good biochemistry background and coming from more of a veterinary background, this was not readily apparent to me. My idea of protease was an enzyme that catalyzes the breakdown of protein, so you think like in your GI tract, you have proteases that break down proteins into amino acids so you can absorb those amino acids and use them as building blocks in your tissues. What I did not realize was that proteases can also serve to activate other proteins, so this is the zymogen idea for any of you who are familiar with that. Proteases are really powerful at breaking down proteins and you do not want them unrestricted in your body because, as you could imagine, that could be quite detrimental, so the body really tightly controls activation of proteases. It does this by secreting them in an inactive form and then having another protease at the target site act upon them to cleave off a piece so that they can be active themselves. I think the best studied example of this, an example that would be most relevant, especially to the veterinarians in the room, is trypsin, which is secreted in its inactive form by the pancreas and if you, let's say, have a cat with pancreatitis, what has happened is that trypsin is getting activated before it gets to the small intestine while it is within the pancreas and now it is starting to digest pancreatic tissue and cause a lot of inflammation. So, that is an example of how protease regulation is really important and one role of proteases is to activate other things within the body and viruses take advantage of this.

So, how do viruses do this? Viruses like our coronavirus, their spike protein is in an inactive form on the surface and I say it is inactive because the fusion protein, the region that allows it to fuse, is hidden inside this coil here. A host protease comes along and clips this region so that you can have a conformational change where this area, the fusion peptide, is exposed. A fusion peptide is a string of hydrophobic amino acids that can insert into the membrane and disrupt them, so as the membranes get closer, it allows them to fuse and this is a process that cells do not like to do. To fuse two membranes is quite difficult; it requires energy. So, the fusion protein gets in there. You have a conformational change in the spike to oppose. This is the host cell membrane. This is the viral membrane. This is a nice schematic of what happens next. You get this formation of this core where the membranes have fused and this is the viral genome. It is now able to enter the cell and start using the cell's machinery to replicate itself. The end result is infection.

Viruses can modulate what cell type they are going to infect by controlling what protease activates them. They do this by selecting certain amino acids at their active site. I think the best analogy I have come up with to explain this is a lock and key analogy, where a coronavirus, at the region where it needs to get activated, has an amino acid sequence that is only going to fit a certain key. For the example of trypsin, which is that protease we talked about in the gut, that likes to see a lock that has one basic residue. Basic residues are positively charged. This is a nice electrostatic interaction for the protease. Trypsin can come in and recognize this basic residue and cleave. So, if a virus has a motif that can be activated by trypsin in the gut and only by that protein, it can restrict its activity to the GI tract. As you can imagine, if a virus is active to infect any cell it encounters, it is going to infect the very first cell that it meets in the body or if it cannot replicate in that cell, that is not its target cell, then that is a dead end for the virus. The virus is much better served to target itself to the tissue where it can replicate in this instance the gut, get activated there and then productively have infection in the GI cells.

I am sorry. This is not showing up very well. For those of you who did not get a color copy of this printout, I will leave you my e-mail and you can e-mail me and I can get you a copy of it printed out because some of this is also very small and hard to read, I realize that.

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Furin is an enzyme that is present in every tissue in the body and I think of that as like the master key. If the virus wants to use a master key, it has to have a lock that is going to be opened by that master. So, the sequence for activation by furin is a multibasic residue. So, these are amino acids that are positively charged in a multibasic string and Harrington recognized that and cleaved that. The best studied example of viral pathogenicity that is linked to cleavage activation is avian influenza. So, you have low path avian influenza that has this monobasic one arginine activation site in its spike. It is cleaved by trypsin in the GI tract. It is really limited to the GI tract in birds and that is what avian influenza is naturally in birds; it is a GI disease in birds. Then you can have the evolution of high path avian influenza virus and I am sure you have seen in the news that there are definitely some high path strains out there that are very fatal in birds and do spread systemic. What has happened in those viruses is that they have acquired this multibasic activation site that can be recognized by furin. I have told you furin is that master key; it is in every tissue. Influenza is capable, actually, of replicating in a lot of tissue so what happens in birds that are infected with this virus is the virus spreads systemically and you have death of the birds. So, with that in mind, we are going to approach the problem of feline coronavirus because it is a similar story. We are talking about a virus that, in its benign form, is restricted to the GI tract and in its pathogenic form, it is capable of infecting macrophage. So what is going on?

I have already introduced to you the coronavirus genome and I told you that we know that mutations in accessory proteins are associated with the development of FIP. The problem is we cannot really link how mutation in an accessory protein would explain a switch in tropism or ability to infect. Tropism is just like what tissue the virus can infect from the GI tract to macrophage. We know that a mutation at a viral activation site can explain that for other viruses like avian influenza or Newcastle disease. So, in our lab we took the approach where instead of looking at the entire genome, we were going to focus in on this viral activation site and we were going to sequence activation sites from cats that had FIP and cats that were healthy and we were going to compare the activation site between those two groups of viruses. Our hypothesis is that FECV and FIPV have different activation motifs, that they are activated by different proteases and that this difference in activation targets FIPVs to monocytes and macrophages and enhances its replication in this cell type. So, when I had the opportunity to design this study, I had just taken epidemiology as a first year vet student. This was exciting for me. I wanted to have a pretty rigorous study and I decided we were going to need to have cases that we knew were FIP that were confirmed by the gold standard, which is immunohistochemistry. So, what we did is we collaborated with Dr. Duhamel, our pathologist, and we found cases in the pathology archives where we had IHC-positive cats, and then we took our controls from environments where we know there is a lot of coronavirus, so these are healthy cats that came from shelters and catteries where they were probably exposed to coronavirus and were not showing any signs of FIP that seemed to just have benign run-of-the-mill coronavirus infection. From these cats we had feces and we went ahead and we sequenced the virus from the feces and the tissues of the cats to compare it. When it was all said and done, we had 30 control cats and 11 FIP cases. We tried to get a mix; we got some wet cases, some dry cases, and mixed cases. We were hoping we would see differences between wet and dry FIP based on the virus. I can tell you right now, we did not see that, but that does not mean that there are not differences. There were not differences in the area we were looking at.

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Our geographic distribution

We wanted to make sure that we were not just describing coronaviruses that were limited to New York State and the area around Cornell, so we tried to take from a really diverse segment around the United States, so our cats came from all over the US. We had samples sent to us from vets, from cat owners. We had the samples from the pathology archive.

The methods were real simple. We isolated viral RNA. We used a method called reverse transcription to turn that RNA into DNA. You can amplify DNA by the polymerase chain reaction, then you can take that amplified DNA, sequence it, and then we analyzed the sequence we got back.

Before I show you the data, I want to share this picture. This is really beautiful. This is IHC-positive omentum and you can see that the areas where there are coronavirus, the macrophages light up red. This is really beautiful and this particular picture was provided by ANTECH. One of the reasons we had some success with this study, if you are trying to isolate RNA from tissue that has been fixed, which is what we were trying to do with the pathology archives, that RNA is really degraded because formalin really degrades RNA, but Dr. Duhamel went in and he selected and picked out these regions that stained red for coronavirus antigen, so it really enhanced our ability to get viral RNA out of these tissues.

So, to orient you here, what you are looking at is the S1/S2 activation motif in viruses that were sequenced from the tissue of healthy cats. I think what will strike you is that you can see there is this really high conservation of this very basic motif. I colored the basic amino acids here in blue. This is an ideal furin motif and if you have been following me, you are probably like “Beth, I’m a little confused. You just told me that avian influenza, which is spreads systemically, is cleaved by furin, has a multibasic motif. Now you’re going to tell me that cats with enteric corona, which is restricted to the intestine, also have this polybasic motif?” We are going to get to that, but yes. That is what it looks like. It looks like, for whatever reason, in the GI tract, this region, this multibasic furin motif is really important. This is highly conserved. The virus does not mutate in this site. We only saw two mutations in the cats that we had and it turns out that mutations of these residue are not as important as mutations like here, this first residue when it comes to furin activation.

Okay, so now we are going to look at what is going on with our FIP cases. I think you can see immediately that that conservation has been lost. We had 22 different viruses from 11 cases. So, what we did is we sequenced different tissues and we got different quality tissues from the same cat. I am going to highlight all of these we saw. There is a big difference from the previous slide. In 10, 11 cats the furin motif was completely disrupted by the presence of an amino acid mutation that varied from that consensus furin motif we saw in feline enteric corona. I am going to walk you through the findings cat by cat. In this animal, we sequenced two tissues and we saw a mutation, the same mutation, in both tissues. In this animal, we also sequenced two tissues and we picked up two different quasispecies species. Both had a mutation, but they were in different sites. In this kitty, one tissue had the enteric motif that we saw in the feces, while the other tissue had a mutation. Then, in this cat, the last cat, we did not see any mutation that would correlate with FIP. You can see we have that really nice arginine, arginine, serine, arginine, arginine, serine motif. What we did see here is this phenylalanine and we are not really sure ... Phenylalanine is a pretty big, bulky amino acid and we do not know in the context of the feline spike what that amino acid would do with the ability to get furin in there. You can imagine if you have not site your protease

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needs to access and then you stick something big and bulky next to it, can furin still cleave that site? I don't know. That is a question that we will have to answer with a genetically modified viruses in the lab. We are planning to do those experiments in the future. But the take-home message, really, is that in FECV, you have this conservation of this multibasic motif and in FIPV, in 14 out of 22 tissues we looked at, that conservation is lost.

So, what does it all mean? What we think is going on is when you look at the amino acids that are taking the place of those basic residues, they all have a different character. They are mostly nonpolar, uncharged amino acids and what we think is happening is that FIPV is being activated by another protease, a protease that does not like basic residues; in fact, it is a protease that prefers these nonpolar amino acids and our working model is that use of that protease to target FIPV to macrophage.

This is where we are going to get into those where we are going from where we have been and, hopefully, talk about potential treatment for FIP. I don't know if you guys can read this in the back, so I will read it to you. When you do as much reading as I do in my PhD, you go back to old magazines, and this is from a 1995 journal; I think it was Feline Practice. This is an old ad for Primucell and it reads: He survived the neighbor kid's BB gun, a Doberman, a garbage truck, falling asleep on a car engine and a fall off the roof. Here is his empty collar and it says 'It's a shame he wasn't vaccinated with Primucell FIP.' This is the saddest advertisement I have ever seen. I don't know what Pfizer was thinking but what it makes me think when I see this is yeah, so in 1995 we thought we had the magic bullet. We thought we had a vaccine for FIP and it turns out we did not, really we are looking for that, we are looking for that treatment for FIP because it really is a tough disease and although this ad is really sad, the sad part is that it is a reality. So, like I told you, in Gary's lab we are really hoping that our research will bring us one step closer to that ultimate goal.

We talk a lot about proteases and I just want to share with you that proteases are dysregulated in a lot of diseases. For example, in dental disease you have up-regulation of proteases that start breaking down gum tissue. In pancreatitis, we talked about trypsin; if it is unregulated, it starts digesting the pancreas. In cancer, we have up-regulation of proteases to break down extracellular matrix, so you can get new blood vessels into those tumors that are growing. When you think about FIP, you have infection of macrophages and macrophages secrete proteases to break down matrix. We know that there are vascular changes in FIP, that you do see effusion, and this we suspect is mediated by some of the proteases that macrophages produce when they are activated. On top of that, we are thinking that a macrophage-produced protease may in fact activate FIP. The protease that we have in mind is MMP-9. I have given you the lock-and-key analogy. I like to think of us a little bit like locksmiths. We are looking at FIP and we are looking at all the different locks that FIP has, all the different residues that appear at that cleavage site, and we are trying to make a mold of this key. We are trying to find out what is the protease that activates this virus. Our best lead at this moment is a protease called matrix metalloproteinase 9. It is a metalloproteinase because it has zinc, a metal, in its active site. It is known to be produced by macrophages and endothelial cells to break down extracellular matrix. It has physiologic roles in the formation of new blood vessels, in wound healing and in cell migration, but it is also associated with a lot of pathologies in people. It is up-regulated in arthritis. It is up-regulated in some vascular diseases and in metastasis in cancer. What is really interesting is that it likes these uncharged amino acid residues like the ones that we see in FIP.

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So, our first question is: Is macrophage expression of this matrix metalloproteinase 9 up-regulated in macrophages that are infected with FIP? This is a picture here that I had taken of feline macrophages, so what we do, we never infect any cats with FIP in our work. We have cats that we collect blood from. These cats do not have any coronavirus, so we draw blood from them and then there is a process by which you can isolate macrophages from that cat blood. You can bring the macrophages back to the lab and you can culture them and then you can infect those macrophages with FIP and see how they respond. So, we did that. These are macrophages that are infected with a strain of FIP called 1146. These are our control, unaffected macrophages and then these are macrophages that are treated with a bacterial protein called LPS that we know stimulates MMP-9. So, we have our negative control, our positive control to show that we can measure MMP-9 and we can actually get it up-regulated when we see FIP. So, we have seen up-regulation of MMP-9, as compared to the control, and these experiments were done last year by a leadership student who came to us. He is a vet student from Germany, Hendrik Sake, who is really excellent. What I have been trying to do now is repeat some of his work to get ready for publication.

Another thing he did – and this is really pretty interesting – we have been collecting blood from pairs of healthy housemates and their siblings or housemates that died of FIP, and the idea is that both these cats were probably exposed to the same enteric coronavirus because they were in the same household. Well, one of them ended up breaking with FIP while the other cat remained healthy. So, we looked at the blood from these cats and looked at the expression of MMP-9 in the blood. We found that in the FIP cat in this one pair that we looked at had 1.5 times more MMP-9 than the healthy housemate and this something now we want to repeat with other cats to see if it holds true, that MMP-9 is in fact up-regulated in macrophages. For any of you who are associated with Dr. Kipar's work – she is a pathologist from Germany – she has also looked at MMP-9 and shown that, yes, it is up-regulated in FIP.

Our future work is to answer some new questions that we have. Do elevated levels of MMP-9 in FIP-infected macrophages contribute to the vascular permeability that we see in effusive FIP? And even in dry FIP, you have permeability of the blood-brain barrier because these cats survive long enough now that the virus is able to get into the brain and cause some neurological signs in cats that suffering from dry FIP. We know that MMP-9 causes vascular problems in people but also going on in cats. Does MMP-9 upregulate the migration of infected monocytes and macrophages into tissue? Does MMP-9 enhance infection of macrophages with FIP? And this is really the key question we want to get at because if it turns out that MMP-9 activates the virus, then we might be able to get an inhibitor of MMP-9 to help cats control the infection. This is something that has been done a lot with cancer, so there are actually a lot of MMP-9 inhibitors out there in drug development because they are targets for atherosclerosis and for cancer, so it is something the pharmaceutical companies are looking at and that is exciting because it gives us access to tools that are already out there.

So, this is the future. This is not something we are currently doing, but this would be the idea, is that you could use the inhibitor of MMP-9 as a treatment for FIP. Currently the standard of care in people with viral infections like hepatitis C or HIV is to receive cocktails of multiple different antiviral inhibitors. The reason for that is these viruses mutate, so if you give a patient just one drug, they are going to immediately develop resistance to that drug. We have learned from HIV patients that you need to give multiple viral inhibitors in order to control the infection. I think that if we want to be able to effectively control FIPV, this will also be something we will have to consider. There is probably not going to just be one inhibitor that you can give; you are probably going to want

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to give a cocktail of drugs, so you can suppress viral replication enough that you do not have these mutants that are resistant to your treatment. And then in people already, you can use doxycycline, which inhibits MMPs in periodontal disease. People who have periodontal disease can take this drug Periostat to inhibit matrix metalloproteinases and help control their periodontal disease. So, you know that kind of thing is an option too. Doxycycline is something that we can try as a potential therapeutic in cats. I did talk to Margie (Scherk) last year at ACVIM. She said that she thought they maybe have tried this with some of their animals and not seen any improvement, but it is an idea that is out there and maybe this is not the exact drug we could use, but it is something along that theme.

I did want to say that I think any treatment for FIP is going to have to be initiated really early. The reason for that is once a virus gets into macrophages, once those macrophages get activated and start producing all these proinflammatory cytokines, you are really at a point of no return. I think even if you add in an inhibitor later on in the infection, it might be too late because you have already got the immune response going and it is ultimately that aberrant immune response that kills the cat.

Lastly, I am going to talk about some of our work with ANTECH. I just want to check how we are doing on time. Okay, good. I want to talk a little bit about how FIP is diagnosed and what current diagnostics are out there today.

Clinical signs of effusive or wet FIP. We have obviously talked about the abdominal effusion and this is usually straw-colored and kind of sticky, really characteristic for FIP. The differentials for abdominal and pleural effusion include other diseases like heart disease, neoplasia, cholangiohepatitis and these are things that vets need to rule out when they see a potential case of wet FIP. The other signs of wet FIP are pretty nonspecific. Chronic fever that is unresponsive to antibiotics. Weight loss. Lethargy. A dull coat. And then we can have some changes on your chemistry panels and your hemogram that are not necessarily specific to FIP but can be seen with a lot of different diseases.

Dry FIP has a lot of the same nonspecific signs as wet FIP and then on top of that, some cats will present with ocular changes. The iris will get darker. You can get these white precipitates on the eye, which are keratic precipitates. As we talked about, these dry cases do not tend to progress as quickly as wet FIP, so the virus is in body longer. It is able to cross the blood-brain barrier and you can start to see some neurologic signs of incoordination, seizures, tremors, that kind of thing. I think this is really nice picture from Niels Pedersen's paper right here. You can see the iris has become brown and you see that even the pupil is a little bit misshapen. I cannot really see the keratic precipitates on here. I have looked, but I bet you Niels probably could see them. The problem with dry FIP is it can be really hard to diagnose because the cat does not always present with these characteristic signs. They may just be presenting with the nonspecific signs of FIP.

Diagnosing FIP

I feel like clinicians are really pretty good at diagnosing wet FIP and a) there is a limited number of things that cause effusion, especially if you have a young cat, which is raising your index of suspicion for FIP, and b) when you have effusion as a clinical sample, any diagnostic test you are going to run is always going to give you better

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results on effusion than on blood or another sample. That is just something we know from the literature. A lot of that work was done by Katrin Hartmann. I don't know if she has ever spoken to you all here, but she is really wonderful. She has done a lot of work on diagnosing FIP.

Blood-work-wise, if the effusion has this kind of albumin/globulin ratio, something less than 0.9, if you have a high total protein, (Oh, I am sorry. This is not blood work; this is if you are looking at effusion), if you have elevated gamma globulins. If you have coronavirus antibodies at all, any titer, and an effusion, that is pretty suggestive that it is FIP. If that effusion is positive for coronavirus by RT-PCR, pretty suggestive that it is FIP. If you are in a place where you can do IFA, which is immunofluorescence assay, on macrophages and you see virus in the macrophages, that also raises your index of suspicion that you have FIP. So, for wet FIP veterinarians have a really good handle on that.

There is also this Rivalta test and what does this mean? This is a paper from Katrin Hartmann, the vet I was telling you about, who has done a lot of work on diagnostics. So, the Rivalta test was developed in the 1800s by a human physician who wanted to be able to tell transudates, which are low-protein effusions, from exudates, which have a lot of protein. To do this, you take a little bit of vinegar, you mix it with some water, and then you draw up a bit of the effusion on the top of that and it looks like, I don't know, eggs benedict, you use vinegar because it helps the protein spread out, so if you have a transudate, as soon as the effusion hits, it is going to spread out. You are not going to see this pellet form, but if you have an exudate, then you are going to see this sort of thing, where you can see there is a pellet of protein and it is diffusing through. The Rivalta test, like for shelters, is really cheap and you can take a little bit of effusion and you get a positive Rivalta, that increases your index of suspicion you have FIP.

Okay, now, if you have dry FIP, then you really are stuck looking at blood, so you are going to look at serum and tests on the serum are inherently a little less accurate, but there are some parameters that we know are associated with FIP in serum. People ask us a lot in our lab, "What about the feline coronavirus antibody test?" They will call us and they will tell us they have a cat that has really high antibody titers to feline coronavirus. Does it have FIP? I think that it has really been shown that the antibody test is not really a good indicator of whether a cat has FIP or not just because so many cats are exposed to enteric corona and do develop antibodies. However, people thought maybe we could use it to rule out FIP and the problem with that is that in FIP, you have formation of antibody and antigen complexes, so antibodies can start to bind up all the coronavirus antigens in blood, so if you are going to go look for a coronavirus antibody, you might not see it because it is all bound to the antigen. We know that titers decrease in some cats as the disease progresses, so you can have a cat with a totally negative antibody titer and 10% of the time that cat is actually going to have FIP. All the antibodies are going to be bound up in the antigen. So, that is neat and that comes from more of Dr. Hartmann's work.

For those of you who are familiar with the current RT-PCR test for coronavirus, there are a couple out there, but the one that I am going to talk about is ... I think the place that does most of it is Auburn University. This test is based on looking at a gene that is only present with coronaviruses that are replicating, so trying to isolate or amplify that gene. So, if you have coronavirus replicating in the blood, you are thinking FIP, right? So, they will look at blood and if they see coronavirus replication by amplification of this gene, it is going to be positive for this test. This was developed in 2004 and the published values have a really good sensitivity of 94% and

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specificity of 100% on blood, but I am going to tell you, just from my experience in our lab trying to isolate coronavirus from blood, just the RNA itself, I do not really believe this. It is actually really tricky to do and we have had a hard time with it in our test at ANTECH so I think these numbers need some independent verification, but certainly ... and, you know, it is a nice test. You can submit ... the sample that works best is effusion. I have told you that already; if you have ascites, that is the best diagnostic sample. You can also submit fine-needle aspirates or you can submit on blood and then you can get an idea whether you have replication. So, this is just another test you can run in those tough cases where you are not sure what is going on.

Okay, so, I wanted to provide this for you guys. It is just a flow chart on FIP diagnosis. I adapted it from one that was published in 1993. So, you know the signs of FIP, if you have those, combined with the risk factors and when you do your clinical exam, you see effusion and that effusion is positive on the Rivalta test and you are thinking, "Okay, we have FIP." If you do not have effusion or you have an atypical effusion ... let's say you have some of those other signs of FIP, the ocular signs, the neurologic signs, then you are going to go ahead and you are going to do some lab work. If they come back and you have a lot of abnormalities, like greater than six abnormalities on your lab work, then you can say, "Okay, we're in the category; this is probably FIP." The problem is when you get those difficult-to-diagnose cats and their blood work. They do not really have the typical effusion. The blood work comes back without as many abnormalities as you might expect in an FIP case. What do you do with those animals? Previously what we did was serology and if they had the highest titer, you would say, "Okay, FIP is really likely. Let's go ahead and biopsy." The problem with biopsy, that is pretty invasive and most owners and vets do not want to put a sick cat through biopsy to confirm whether it has FIP, so if you go to places where yeah, it would be really nice to have a blood test for FIP that you could submit. So, Auburn has their test that could be useful in that case. We are working on a test with ANTECH that could be useful in that case for diagnosis. It is based on qRT-PCR. Instead of looking for a gene that is present during replication, what we are looking for is our mutation. So, our idea is that you could submit blood. We are going to look for coronavirus. If it turns out to be positive, we are going to sequence that coronavirus and we are going to tell you whether you have a mutation at that S1/S2 site that is associated with FIP.

Here is the data that we have. This test is kind of a two-step test. The first thing we are going to be doing is PCR for coronavirus. We want to see how good are we at detecting coronavirus in the blood and it turns out, in the blood, we are not very good at it. Our sensitivity in blood is really not ideal yet. So, we decided we are going to start, as we are designing this test, with something that we can do better at. We are going to start with ascites and in ascites we are pretty good at detecting coronavirus. We can detect it in 72% of the cats that we believe have FIP and the specificity is 100%. We also looked at effusions from cats that had heart disease that they had hepatitis or some other reason they had that effusion. We did not detect any coronavirus in those cats, so we feel really nice about the specificity. When we go look at these positive cats and we sequence them and we look at what we find, we find that sequencing itself, 79% of the time the cats that we suspect FIP, we find that FIP would be like mutation, and then specificity in cats that are healthy and we go ahead and sequence the virus, we find 93% of the time that comes back negative for the mutation.

So, that is where we are at with this test now. It is still in development. I am under the impression from ANTECH that they intend to market this test within the next year. But I want to give you my perspective on how I think this test could be used. With a diagnostic test, my concern is that it is really specific because you do not

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want to give a cat owner a diagnosis of FIP when their cat does not have FIP because that, in many cases, could mean euthanasia. And then what are you going to do if you get a positive result? A lot of owners were going to do the same thing anyway. They are going to give the cat as much palliative care as they can until it is time to euthanize that animal.

Where I see this testing really useful is when we get to the point where we have a treatment for FIP, so in that case you could go into the cattery that is having a problem, you could take blood from those cats, and you could look to see which sets of kittens, which queens, have virus that contains a mutation that we know is associated with the development of FIP, and then you can initiate a palliative treatment or not ... You know, you can initiate your treatment in those cats, let's say, for example, you treat them with doxycycline, which right now is not a treatment, but just as an example. You could start treating those cats and, hopefully, prevent that mutated virus that we see from actually causing FIP in those cats; just giving them a little boost to help their own immune systems take control of the virus. So, that is where I see this diagnostic test being super-useful.

I have also talked to one of our shelter veterinarians and she was saying it could be nice in a shelter setting where you have a kitten that has FIP and now you are wondering what to do with the littermates because for shelters, that is a really tough call. Are you going to adopt out littermates of an FIP cat and then have ... we know those cats are more likely to break with FIP and that is heartbreaking for the owner. What do you do with those kittens? Then maybe you can get this test on board and can form your decision or you could even start treatment if you have it available. So, those are my ideas for the future. It is really exciting to work with ANTECH on this test.

In summary, RT-PCR-based tests like the one we are developing are useful tools to aid in making a diagnosis in challenging cases, but IHC biopsy on tissue remains the gold standard for diagnosing FIP. I really wanted to end with some pictures of the cats that have been in our study. We know from owners these are their babies and we definitely really think about them and how they would do in the lab and some of them are really pretty adorable. This last cat, Miles here, was the first kitty to participate in our study and I think he will always stick with me for sure. It is really heartbreaking to get pictures of these guys. They are usually kittens that are usually so adorable and it would be great to have a treatment for FIP.

All right, thank you. This is my contact information, so please feel free to e-mail me... [applause]

END OF THIS AUDIO SECTION