



WINN FELINE FOUNDATION

For the Health and Well-being of All Cats

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37th Annual Winn Symposium – July 2, 2015

“What’s New Pussycat – Advances in GI Therapy”

Transcript of Audio: Introduction and Dr. Jody Gookin on Kitten Diarrhea

Introduction

Steve Dale:

So good afternoon, welcome to the Winn Feline Foundation Symposium. How many of you have been to one of our symposiums before? Most of you. How many haven't? Well can everyone applaud for those folks that haven't, and we'll say welcome. How many of you happen to have a cat? Alright now two people in the room didn't raise their hands, as well as the bartender. So what I want to know is why those three people don't have a cat, and I do know that we have the Toronto Humane Society in the room, so we can hook you up by the way. Pretty much everything and most of you in this room I think probably know about the Winn Feline Foundation.

My name is Steve Dale, incidentally, and I have been on the Board of the Winn Feline Foundation now, I think for about 10 years. I have been moderating this, the Winn Feline Symposium for 5 or 6 or 7 years. It is always an honor for me to do, and it will be again today. I have the opportunity to introduce two of the best veterinarians in the field, in the world, literally on the planet, at what they do, and for us to give exposure to those veterinarians is an honor for us to at the Winn Feline Foundation, and hopefully you will find it useful, and appreciate the fact that these are superstars, and they are superstars in veterinary medicine. Are there veterinarians here? Great! So for you veterinarians, fill out the comment card that you got from Janet, and if you don't have one, Janet raise your hand back there the back of the room, and you can get one and fill that out, and then you can get your CE certificate back.

Talking about the Winn Feline Foundation, so I asked how many of you have a cat, and except three of you, you all raised your hand. So for those of you who have a cat, who knows someone who has cats, everything pretty much about that cat, that goes on with that cat, from the morning when the cat wakes up, well let me put that a better way, wakes you up is probably a better was to put it, until the end of the day when the cat finally allows you to go to bed. Everything in between all that, the Winn Feline Foundation has probably funded studies about, to what our cats eat, to the vaccines they get, to the health report that a veterinarian gives them every time they go to the veterinarian. We are not only talking about pedigree cats, and breed-specific illnesses, and we are, we are talking about all cats. The Winn Feline Foundation for nearly 50 years now, has had something to do with it. These symposiums have been going on for decades. Joan Miller is the lady who said, in her head, somewhere, somehow, I am going to start these symposiums. We are going to bring education to breeders. Joan isn't here this evening, but I still wanted to acknowledge her, a round of applause for her please.

We truly can't do it without my colleagues on the board I suppose, so for those colleagues on the board, I am very proud to be on this board all these years, I ask you to stand up. There we go, the Winn Feline Foundation Board of Directors, and our Executive Director is Dr. Vicki Thayer. Dr. Thayer helped organize this event. Dr. Thayer is past President of the American Association of Feline practitioners. She is a superstar in veterinarian medicine, we are grateful to have her, and boy does she work very, very, hard. It is not only her, it is our grant reviewers who are all experts in feline medicine that say, I wish we had more



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money. Every year it happens, we want to support more grants. We want to support more studies, there is good research out there, and every year we don't have as much money as we would like, and that is worked all of you can come in.

I am not asking you for donation, well yes I am asking you for a donation, but it is really you telling your friends, you telling people you know about the Winn Feline Foundation. If you are a breeder, someone purchases your cat, it would be great to tell them about the Winn Feline Foundation. You know what, we have two very specific ways people can donate. There are two funds, one is called the Ricky fund, which I have something to do with. That is for cats with hypertrophic cardiomyopathy, so that we can better understand that heart disease in cats. The other is for FIP, feline infectious peritonitis, that is called the Bria Fund. When a cat sadly passes away for you veterinarians, but also for anyone, when a cat sadly passes away from one of those illnesses, even if you made a \$5 donation, \$500 would be better but really \$5 is fine, it is acknowledging to your client that this happened and I am making a donation in your cat's name to the Winn Feline Foundation, that is a great thing that helps all other cats helps of course the Feline Foundation, and that cat owner knows you care and this is all available on our website, which is winnfelinefoundation.org.

Before I introduce my first speaker, you know what, I am just talking, I am very bad for this. This is our mission, I talked about all this already, I am not even following what I am supposed to be talking about, but you can read and most of you know about it the Winn Feline Foundation, and yes, this is sort of hit list. If we had David Letterman here to count off the top 10 list, this would be top 12 maybe, of just some of what we have done. If you can read what they are, some of them are recent, some of them are not quite as recent, I will point out one or two. In the 1960s, cats were dying of dilated cardiomyopathy, a kind of heart disease and also getting blind, and it is like why was that? A veterinarian from the University of California, Davis, Dr. Paul Pion said, "I think I know why. I think there's not enough taurine in cat food." Some other organizations thought he was crazy, and we said, well you might be crazy but this actually makes some sense, despite your personality. If that is tweeted out you are in big trouble, he is a nice man. What I am trying to say is that, we said yes when others didn't, and it turned out he was right about that. Feline asthma was not very well understood until actually quite recently, and it was the Winn Feline Foundation that that funded those studies. If you are a Maine coon or ragdoll breeder, you know about the genetic markers for those cats for HCM, hypertrophic cardiomyopathy, and that was funded by the Winn Feline Foundation, and this is a very, believe me, small list.

Now, I ask you to turn down your cell phones if I didn't do that, I am supposed to say that. We have cards, comment cards, question cards the index cards on your table. You can fill those out, and this is the way to ask questions. I will read those cards after both speakers speak. Also we have these beautiful books that we made up for our 40th anniversary, we are close to 50 years now, so we have a lot of these left in our storage unit, between me and you, so please feel free to take any or if you have cat loving friends, relatives, family members, other breeders that would want these, veterinarians, technicians, please take these back to your homes, your practices, we appreciate that.

She is a superstar in veterinary medicine, we have funded Dr. Jodi Gookin many times. She is an associate professor at North Carolina State University. I will tell you, I speak at many veterinary conferences, I go to Veterinary conferences all over and there aren't many veterinarian conferences where Dr. Gookin's name is

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not there, and you are not getting one or two or three talks, but you are sometimes giving four or five or six talks, and she is as I said, a star. We know more about understanding the gut, when I say that of cats, because this lady has had the guts to do it, Dr. Gookin.

Beginning of Dr. Jody Gookin Audio

Dr. Jody Gookin:

Alright, can you hear me? Well it is I that am proud and honored to be invited by the Winn Feline Foundation to speak to you today. What I am going to talk to you about is something that is near and dear to my heart, and it is something that would never have happened if it wasn't for the Winn Feline Foundation. It is a work in progress, but we stick with what we start, and we are going to help kittens with diarrhea and kittens that are dying with diarrhea. We have been going through this journey, looking at bacterial causes of diarrhea in young kittens, and we have, we think we have discovered an important bacteria that causes diarrhea and might kill young kittens due the diarrhea, and another that might even protect them against that diarrhea, and that death, but we are still in the process, but I would love to share with you this story.

First of all, why is what we are doing and what the Winn Feline Foundation is doing important. In the U.S. alone, there are 74 million owned, and 70 million feral cats. Each year, they give birth to 180 million kittens, and an inestimable numbers of these kittens end up at one of 4000 to 6000 U.S. animal shelters. In those shelters, it is estimated that 15% of those kittens will die or be euthanized due date illness before they reach 8 weeks of age. What is disheartening about this, is that an obvious cause is unknown in as many as 50% of these kittens, and hardly anybody seems to care, or has spent much time studying what is happening to these babies and why they are dying.

The few studies that we could actually find that look at mortality in kittens, we can find that the weaning period seems to be the most vulnerable. This study was published in 2002, out of the UK, and it was a necropsy study, so post-mortem examination of kittens that had died, and there were 274 of them, and actually these 4 kittens died in the perinatal period, 38 neonatal, 30 pre-weaned, but 202 post weaning. So it is that weaning period that seems to be very critical, and we think that is because of the stress of weaning, there may be a role in failure of immunity transfer from the queen to the kittens, crowding, sanitation, changes in diet. What do we know? We know that infectious disease appears to be the most common cause of death in these kittens. So these yellow areas are the percentage of kittens that died from an infectious disease and among those infectious diseases, 71% of them were viral, and we are looking at a lot of death from panleukopenia. So that is an area that needs some work, and 20% of those were actually bacterial causes of death. The other thing we know from these studies, is that these kittens often die with clinical signs or post-mortem evidence of intestinal disease.

So they die of vomiting, diarrhea, and we know that infections are common in these kittens, so it makes sense that focusing on infectious causes of diarrhea or GI disease in these kittens seems like a high priority area. The tragedy extends to the fact, and I am never lacking for amazement about this, that diarrhea is the second leading cause of childhood mortality in developing countries. So 8000 children per year die from diarrhea in developing countries.



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There was a recent study done by the Bill and Melinda Gates Foundation and they demonstrated that among the infectious causes of diarrheal death in these children, most of them are caused by a mere four pathogens, and one of these is rotavirus, one of these is a protozoan parasite called cryptosporidium, and the other two are bacteria. Interestingly, these same infectious causes of diarrheal death in children in developing countries, cause diarrheal death in the children of our animals in the United States, our calves, our kittens, our puppies. So that sort of developing country is recapitulated in the environment that a lot of our young animals are growing up in.

So we have two bacterial causes, and in fact if you look at all of the infectious causes that they found in these kids, you will see that diarrhea caused by E. coli bacteria, was significantly associated with a hazard of death. So they found a bunch of different types of infectious diseases, but the two that had an increased hazard of death happened to be E. coli. We all have all have E. coli in our GI tract. Most of that E. coli is good E. coli, but there are bad E. coli in there, and what makes a good E. coli versus a bad E. coli, is the presence of, in the chromosome, genes that make proteins that are toxins, or do bad things, and it is difficult to tell those good E. coli from those bad E. coli and we are going to go into that more.

What we do not know is very much about the role of E. coli as a cause of diarrhea in kittens or cats. What we do not know really much about anything about bacterial causes of diarrhea in cats or dogs. We as veterinarians only think of about four different bacterial causes of diarrhea when we see a dog or a cat, and those are salmonella, campylobacter, Clostridium perfringens and Clostridium difficile. The reason why those are the only ones we think about, well that problem is going to become clear, but we not only don't want to look for them, but we often cannot afford to look for them, because they are really cumbersome to diagnose. So if you want to diagnose an intestinal bacterial infection, what do you do? You start with feces, and you have to culture it on a Petri dish with media that fosters the growth of all the bacteria in there, and then you have to sort of pick a couple of those and find out what bacteria are those, and then you run each of them in a machine at \$50 apiece, so they you can find out that is an E. coli, and that is an E. coli, and that is an E. coli. Okay we all have E. coli. So what is that mean?

Well to know what that means, I have to isolate those three different colonies, well there is a lot more on there than the three, right? Isolate those three colonies, take the bacteria, extract their DNA, put it in a polymerase chain reaction test, run the products of that test out on a gel, to see if those have the gene that codes for a virulent factor. That right there took a minimum of 3 to 4 days, and probably \$150, and that is in house, right. So by that time, you cannot afford it, you are on a fishing expedition, and your patient is either dead or got better despite you.

So what makes it horrible, more horrible about finding bacterial causes of diarrhea, is what we now know about the gut microbiome, the bacteria that live within our bodies. In a human, 100 trillion bacteria in your GI tract, that represents over 100 species of bacteria that carry 3 million genes. So what do you think? Seriously? I am going to put little bit of poop on that dish, and I am just going to select three colonies, and I am going to find the bacterial pathogen. Well if you compare this microbiome to you and me, here is me, one human, one species, I have 23,000 genes, so guess what? I am only 10% human, only 10% of me up here is human, 90% of me is bacteria. So you have tons of these bacteria in the lumen of the gut. There are also other toxic things in the gut with the bacteria, and the only thing separating you from the 90% of other people that are there, these bacteria, is a single layer of intestinal epithelial cells. So there is a lot of



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potential, these guys are good guys, but there is a lot of potential for some of these guys to be bad guys, right. So how are we going to figure out who is bad? Which one of you guys is responsible for the diarrhea, raise your hand. It is not easy to accomplish. Therefore, very few bacterial causes have been identified, and there are so many potential causes that are sitting there, and the ones that we do know about actually vary from really common, to rare, sometimes are present in healthy cats and sick cats, and there are numerous tiers of tests and diagnostics levels you can do, so it is not very easy to get a hold of.

So why the heck would I start going to look at bacteria? I mean that is like a career ender right there, right? Go looking in that for something that might cause diarrhea. So the long-term goal of our work has always been to identify life-saving preventative or treatment strategies that help ameliorate the morbidity and mortality of gastrointestinal illness in cats. My training was as a GI person, not necessarily an infectious disease person. I am more interested in the cat than that bug that is in there. So work that we have done has centered around the relationship between pathogens and that intestinal epithelial lining. So most of you, if you have heard of me before, think of tritrichomonas, and that is a pathogen that's in the GI tract of the cat and it gets all up and close and personal on this intestinal epithelial cell that I have kind of drawn here, of one intestinal epithelial cell with its little nucleus.

We also do work on a pathogen called cryptosporidium, which is also a pathogen that sits on the top of the intestinal epithelial cells. So I guess I like things that like to sit on the intestinal epithelial cells. I like pathogens that like to go to the intestinal epithelial cell and get on it, which is inappropriate. These cells do not want to be touched, don't touch me, don't come near me, you're bad. So it was with this sort of background in mind, that I was asked one day to consult on a case. It was a pathologist from across the road, they got a kitten, and this kitten they wanted to consult on tritrichomonas. It was a 4-week-old Persian kitten that developed diarrhea 5 days ago, became inappetent and died. They did a post-mortem on this kitten, which is always a good idea by the way, to get a post-mortem on the kittens when you lose them, even though I am going to tell you usually don't find anything, still we need more help, we need more data.

They were interested in the tritrichomonas, and had lots of questions about tritrichomonas, but what interested me was what I have underlined, what interested me was that in the gut, in the small intestine of this cat, there were large numbers of bacteria that formed palisades along the intestinal epithelium, and they attached and effaced the epithelial cells. That is a buzz term, attaching and effacing for E. coli. For an E. coli that is abnormal in its virulence factors, and it gets all up in personal, and attaches on these intestinal epithelial cell. I am like cool, that is interesting. I like cats, I like infectious diseases and gut disease, and I had actually never heard of that before. I am like E. coli, like this kind of E. coli can happen in cats? I talked to pathologist, and they said, yeah, I see that sometimes. I am like wow, that is really cool.

I am going to put together a case series, give me all the cases you have ever seen. There were not that many, they came was seven, I will have one of my residents summarize the cases, we will take some pretty pictures, and we will get the word out there that E. coli can be the cause of diarrhea in cats. So we did that. First what we did was we read about what do we need to know about attaching in effacing E. coli? We learned that these E. coli attach to the epithelial cells, the green is the surface of a single epithelial cell that lines the gut, and the bacteria get all up and close to that plasma membrane. The plasma membrane actually comes up and worships the bacteria on this thing called a pedestal. It sounds like a good deal if you are one of the bacteria, you sit there and get worshipped. The way that they do that attachment, is that they have



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this receptor on them called intimin, and the reason why I am telling you this is because the gene that encodes that intimin, that allows that to sit on that cell is called EAE, and it is an important gene that we are going to be looking at as we go forward.

The other thing that they do is they efface the microvilli. Microvilli are like little hairs on the top of every intestinal epithelial cell, and if you look down on the top of the epithelial cells, this is a single epithelial cell, and this is another one, and another one, and you can see these fine hair-like projections, those are microvilli. On those is where the nutrient transporters are, that is where the gut absorbs nutrients and food, and when these bacteria here in increasingly higher numbers, here is a matt of them right here, they efface and flatten out those microvilli, which results in the intestine not absorbing normally.

So you get this malabsorption, and you get this diarrhea, and basically the kitten starts to starve and get dehydrated from diarrhea. There are basically two types of E. coli that do this kind of thing, one of them is called EHEC, and that makes toxins, and that is the E. coli that kids get when they go to petting zoos. It is carried by cows, it is O157:H7, so that one is nasty. So that one, EHEC, can sit on the intestinal epithelial cells like that, and then it makes these toxins. Then there is this other one called EPEC, enteropathogenic E. coli, and it also sits there, but it doesn't make toxins. These EPEC is going to be the one that we are going to be interested in, it is the one we are going to find, but this EPEC actually is one of the ones that is significantly associated with death of children in developing countries, that particular organism, this EPEC.

So we went ahead and we said, give us the cases you have of this E. coli that attaches and effaces, and we will have a look at them. So they gave us 7 kittens. So we got the kittens, and what the kittens were, were little blocks of paraffin with tissue in them. That is all we had, what was left over of this necropsy of these kittens. So we made slices, we looked at them under a microscope, and you can see, this is the intestinal epithelial layer right here, and this is the lumen where the bacteria would live, and all of this purple stuff right here are bacteria that are adhering to these intestinal epithelial cells.

When you Gram-stain that, you can see the bacteria right here, they are Gram-negative. They are negative because they don't look any different than anything else, right? Gram-negative means they do not take up any stain, and then when we look with a transmission electron microscope, we can see these E. coli sticking to these microvilli on the top of these epithelial cells. That was truly fascinating. What we did to figure out, who this is, is this EHEC or EPEC, is to carve out of that tissue with a scalpel, the organisms, extract their DNA, and do PCR on them and figure out what their virulence attributes were. They had EAE, which means they are an attaching and effacing E. coli. They didn't have the Shiga toxins, so they were not EHEC, they were EPEC.

So here we have a demonstration of an important E. coli, attaching to kittens that have died with diarrhea, the same type of E. coli that kills kids due to diarrhea in developing countries. What was the real game changer, direction changer for us, was that we saw this in 2 of the kittens, but in the other 5 kittens, when we got to the Gram-stain, we went what? Because this is what we found. So the pathologist told us that these were E. coli, based on this stain here and I believe him, the organisms are there, organisms are there. Then you do the Gram-stain, and go what? That is Gram positive, that is not an E. coli, that is something else. I have never even seen anything like that before, and when I look with the electron transmission microscope,



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it is doing the same thing that the E. coli does. So more kittens had this Gram-positive thing, than had this Gram-negative thing, whoa, what is going on here? Both of these are inappropriate.

So what we did was we took the scalpel and we carved out the guy, and we did PCR on him and we found out that he was an enterococcus, and a specific species of enterococcus called *Enterococcus hirae*. So at that point, we were like, well what do we know about enterococcus. I don't know anything about enterococcus. So let's look at the literature. This is our kitten, one of our kittens, one of the most dramatic examples. Like this is the intestinal epithelial layer, in a kitten's small intestine. Look at that, and that it is just remarkable. Those bacteria are not supposed to be there like that. It is inappropriate. I am thinking, hey, maybe we found a new pathogen of cats. What do I know? I found when we looked, that this actual activity of enterococci has been described in neonates of many domestic species, but not kittens. Chickens, foals, piglets, puppies, people, little descriptions here and there of this. In those descriptions, the association with GI disease is unclear. Everyone that looks at that is going to say, ewe, that is a pathogen. That is not supposed to be doing that. As we found, for the first time, no one has ever even made this connection before, it is nearly indistinguishable from EPEC infection, in the way it appears under a microscope. The thing in enterococci in general, is they are considered gastrointestinal commensals. We all have enterococci in our gut, just like we have E. coli. That is what makes it so difficult to identify these pathogens, because they are normally present, and they are frequently administered as probiotics. So in fact FortiFlora is *Enterococcus faecium*, that is a species of the enterococcus. I also am aware that the enterococci can be serious potential pathogens, so when enterococci get out of that gut and they get in the reproductive tract, or the urinary tract, or the biliary tract, or the lungs, they can cause disease, and really bad disease, and they can kill cats. They also could be antimicrobial resistant, they persist the environment in the form of biofilms on things that can be transmitted to other animals. What we don't know though, is could they be pathogens under some circumstances? So particularly in a neonate, under the conditions that we talked about, during weaning.

So this is when I went to the Winn Feline Foundation, and I said, I need your guys to help. I think we have something going on here. We have this EPEC, and we have this *Enterococcus hirae*, and they are both attaching seemingly inappropriately to the intestines of kittens. I think we are onto something here and I need help, and that is pretty much what the Winn Feline Foundation has always been for me, somebody that is willing to think outside the box, and invest in the future of something that is not certain, right? We are going to take some risks, we are going to follow the trail, and see what we get out of this. So our objective with this grant was to determine the association between these enteral adherent, these attaching bacterial infections, and terminal illness in foster kittens. Now we looked at terminal illness, because I can't look at this in a live kitten. In order for me to look at whether these are attaching to the gut, the kitten has got to be dead, right. So let's start by looking at kittens that died, and see if we can find it.

So Lenovo ThinkVantage Toolbox stopped working. [LAUGH] So our aims in this study were to define, what is the healthy enterococcal community in the gut of foster age kittens. I don't even know who is supposed to be there. Are you supposed to give them FortiFlora? What is up with this E. *hirae*, I have no idea what we are working with. We focused on kittens that were under 12 weeks of age, under 1 kg body weight. We looked in the small intestine, because that is the workhorse of the GI tract,



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its most important part, and we looked at the bacteria that were up close and personal with the epithelium. We wanted to know, how is that microbiota impacted by severe illness in this population, and is there any association between the presence of these attaching bacteria, and kitten mortality? Our approach was two groups of kittens. To get healthy dead kittens, we were able to work with our County Animal Shelter, who at the time had a very poor ability to foster their over slot with animals. It is hard to get kittens from them anymore, thank goodness, but at one time they had to kill a lot of kittens, because they had no place to put them. They allowed me to have those kittens and necropsy them and use them in this study, and we got 50 from them.

The other 50 came from the SPCA, lovingly cared for it, fostered kittens, who either died unexpectedly, or were dying and humanely euthanized. So that is our population. Each of these kittens, one at a time, were brought to NC State, and we did a full autopsy. We removed the entire GI tract; we took selected pieces of the GI tract and looked at them under the microscope. We Gram-stained them, we did special techniques called fluorescence in situ hybridization, that would make our bacteria glow if they were *E. coli*, or glow if they were enterococci, and we carved out the organisms and looked for the genes, like we did before, but we also took the small intestine and we cultured the surface bacteria for enterococci. We gathered the enterococci, we figured out who is there, what types of enterococci, are they virulent or not, and what is their antimicrobial susceptibility? Here is our kitten populations, 50 healthy kittens, 50 sick and dying kittens. They all meet the selection criteria, so their average is about 6 weeks of age, equal male and female, body weight averages 360 g in the sick kittens, significantly lower than in the healthy kittens, because they were really, really sick. Also, longer time for us between the time the kitten died, and the time we did necropsy in the ones that were sick, because they died in the middle of the night, whereas the other ones were going to be predictably be killed, and we could be there right away and do our necropsy.

Pre-mortem, the kittens got a fair amount of medications, especially the ones in foster care. So not only dewormers and vaccinations, but oral antibiotics, even probiotics, antiemetics, appetite stimulants and things, whereas the other group was pretty unadulterated, because there was not much invested in them, and they were not cared for for very long. The clinical histories are interesting, because we did not select based on any criteria, other than the kitten died. Here we find that in the sick kittens, 50% of them died with predominantly clinical signs of gastrointestinal disease, which is what we think, that most of these kittens do die with GI disease. The second most popular cause of death, upper respiratory, ocular, viral, another big, big, area we need to put time and energy into. Then a mishmash of wounded, disabled, failure to thrive, and so forth.

What we first did, was we did a routine autopsy, and we looked at all the sections, just with a light microscope, nothing fancy, of the GI tract, the stomach, the duodenum, the ileum, the colon, in the healthy and the sick kittens. I can summarize these pretty easily for you, it is what we already knew from the very few studies that have been done in this population, and that is that you can find minimal clues as to why a kitten died, by just doing a vanilla flavored light microscopic examination of the GI tract. When we hunkered down though, we found some very interesting differences between these two populations, and I want to summarize them for you.



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First let's talk about what we found in the healthy kittens, and then we will talk about what we found in the sick and dying kittens. In the healthy kittens, and in all the kittens in general, we found six different types of enterococci in these kittens, by culturing their contents of their small intestine. Interestingly, we were not expecting this, in healthy kittens, the most popular, so to speak, enterococcus found in the intestinal tract, was *Enterococcus hirae*. That is the one that we found attaching to the villi. So *Enterococcus hirae* is the most popular enterococcus for a healthy kitten to have inside the small intestine.

Moreover, when we looked up close and personal with our special stains, and gram-stains, and fluorescence in situ hybridization, in these healthy kittens, guess what? The healthy kittens were the ones that had the most, and richest, and largest surface area of these enterococci sticking to their gut. I got the grant from Winn telling them this is going to be a pathogen, now I am thinking this is the savior. What is going on? These kittens are full of this stuff, and it seems to be associated with health. If you make them glow, you get really pretty pictures too, all over that villus, attaching to the microvilli. What we did not find in any of the healthy kittens was EPEC. We did not find a single healthy kitten that had that *E. coli* sticking to the gut.

So what about the sick kittens? In the sick and dying kittens it was interesting, because when we cultured our enterococci that are found in that small intestine, all of the sudden we got less *E. hirae*, and more *E. faecalis*. So one term for this would be like a dysbiosis, a displacement, or an asymmetry, or an abnormality in these populations. If you look at it in terms of percent of isolates, in these sick kittens, our friend we think, *E. hirae*, is being displaced by other types of enterococci. When we looked up close and personal at the GI tract of these poor kittens that had died, an example right here of a typical perineal soiling, skinny, cachectic kitten that died, we see that we find Gram-negative glowing in the dark, enteropathogenic *E. coli*, in a significant number of these kittens. Interestingly, some of the sick kittens did have *E. hirae* stuck to their intestines, but none of the kittens with EPEC had any *E. hirae* around.

So how do we interpret this?

Well let's summarize, 60% of terminally ill kittens had either clinical or histopathological evidence of significant gastrointestinal disease at the time that they died, and the majority of it we could attribute to the small intestine. We also found that the healthy mucosa enterococcal flora in this population of kittens is *E. hirae*. We also characterized those *E. hirae*, and we found that they are pretty avirulent, they do not have virulent factors, they are susceptible to antibiotics, they don't form biofilm, they are relatively friendly on the scale of enterococcus.

In sick kittens though, we saw a dysbiosis of those enterococci, and a displacement of the *Enterococcus hirae* with more of this *E. faecalis*, and when we cultured that *E. faecalis*, that sucker was nasty. So the *E. faecalis* in these kittens came from multiple origins, they were very diverse, they were very anti-microbial resistant, they loved to form biofilm, and they made a lot of virulence factors. We are not right now looking at this *E. faecalis* as a pathogen, but I think we should, because we have got too many other things we are chasing, but that guy right there he might be bad, and what the heck is he doing in these kittens, and could he be hurting these kittens, this *E. faecalis*.



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So what it looks like is that enteropathogenic E. coli, definitely in this study, is significantly associated with death in kittens when seen to attach to the gut, right. Whereas enterococcus hirae seemed more extensive in the healthy kittens, and it was lacking in kittens that had EPEC. So the idea was, could the presence of this guy be friendly in a way that discourages EPEC from being able to attach. They seem to be mutually exclusive, so is this EPEC the bad guy, but if you have this guy, you are less likely to get EPEC because there is nowhere to land on the epithelium. So this is our E. hirae, a good guy. Moreover, could this E. hirae be a very nice probiotic in this group of kittens? What you will see is that E. faecium, which is what FortiFlora is, was not something that we found as normal inhabitant of the small intestine of kittens, in the manner in which we looked at it. What we did find is this. There is no commercially available probiotic that does something like that, and that might be that beneficial. It is obviously still very young in development, but we are very interested in looking at this E. hirae as potentially a really important thing that you could give young kittens that are at risk for developing diarrhea and EPEC infection.

So the next step really was, we have shown that in the dead kitten that has attaching EPEC in the gut, that that is a bad thing, right. That is associated with the death, but what we don't know is, how are we going to diagnose this? All we have right now is the attaching EPEC in a dead kitten. That is not going to help me diagnose that disease in a live kittens unless I kill it, and I look for it. Well that helped a lot, didn't it? I need a way to diagnose EPEC in a kitten before it is dead, so that I can try to do something about it.

So what we did then, was started looking at the feces, and using those really laborious old-school methods to culture E. coli, select colonies, figure out if they had virulence factors, and see if we could find EPEC in these live kittens that had diarrhea, and didn't have diarrhea, and kittens that died with diarrhea or didn't die with diarrhea. What we did, was we looked at the presence of not only our EPEC friend here, but all the other classic diarrhea causing E. coli. I mean you are there, you are isolating E. coli, you might as well look for the other kinds too.

So based on a PCR for all these different virulence genes, you can figure out if your E. coli is none of these things, which would be a good E. coli, or it is an enterotoxigenic E. coli, and enterohemorrhagic E. coli, a necrotoxigenic E. coli, or eeblablabla... So we did that, and so far we have got 14 kittens that were healthy at the time they were dead, and 26 that died with clinical signs of diarrhea, and so we are still gathering kittens, but we found pathogenic E. coli in 50% of these kittens, and they fell under the following categories. We found an ETEC and we found mostly EPEC. What we also found a lot of was necrotoxigenic E. coli, which is not the subject of our grant, but should be the subject of somebody's grant, because who knows what those guys are doing in kittens. More of the kittens had NTEC that even had EPEC. NTEC does not adhere though, so it was not the focus of our study.

So here are we are showing that kittens shed live diarrhea causing E. coli, EPEC being an important one. We also know that these EPEC are pretty diverse in these kittens. They have multiple serotypes, so these O and H designations, and when you look at their genetic composition, and you splice up their DNA, and you compare their pieces of DNA to each other they are very different to one another. That means that kittens are getting EPEC from multiple sources. They are not all sharing the same EPEC that they got at the SPCA, they are getting it from the environment, they are getting it from other kittens and so forth and we have even found that some of these sero groups, have been associated with diarrheal disease in kids in developing countries.



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So the next thing we were looking at, and we are concurrently looking at, was. God can we make a diagnosis of EPEC any better than the old diagnostic of EPEC that no one runs anyway? Nobody, like I told you at the beginning, no one is going to culture feces, no one is going to want to pluck colonies, nobody is going to want to do PCR on the colonies. That approach is never going to work to help us save kittens lives in a foster care situation.

So what we simply asked was, I wonder if you could just take the feces, extract DNA out of it, and just look for EAE, because if there is EAE in the feces, then you know that E. coli is in the feces, and you know that there is also E. coli in the feces that makes EAE. EAE was that thing that made them stick to the cells. So can I jump, can I cheat and jump all the way to the virulence gene, and I was talking to Tori, and I am like, it is not going to work, but let's just do it. So we did it, jumped right to PCR. Oh my God, positive, positive, positive, positive, positive, beautiful PCR, one product EAE, cleanest thing I have ever seen, right on the poop. When we did this, we found that this direct PCR detected EPEC in more kittens than the fecal culture did. So the fecal culture detected 8/40 kittens had EPEC, and when we used PCR 21/48, the number is a little different, because not the same number of kittens went into each assay, 44%, so twice as many kittens had EPEC than we found on culture. Moreover, if we compared the number of kittens that had this EAE amplified from their feces, based on whether they were healthy or had diarrhea, the presence of this EAE gene was significantly more common in the kittens that were dying with diarrhea, 58%, than the kittens that weren't, only 18%.

So where have we gone? We have shown that both healthy and sick kittens, so some healthy kittens, shed live EPEC in their feces. Kittens do shed diarrhea causing E. coli in their feces, and EPEC is a really important one. EPEC is more common in kittens dying with diarrhea. Some of them may serve as a reservoir for infection in people, or maybe they came from people. EPEC is potentially very easy to diagnose by PCR, however some healthy kittens, and kittens with diarrhea due to other causes may test positive to EPEC, that is always a problem with these PCR tests, and with of diarrhea there is always going to be healthy animals that can test positive, and there is always going to be sick animals that test positive, but it is because they are healthy otherwise, and there is some other cause for the diarrhea. I know that is complicated, but it is not a litmus test for the diagnosis.

What we think the litmus test for this cat has EPEC, and the EPEC is causing diarrhea, might be that the EPEC in that kitten are actually attaching to the intestinal epithelium. So while healthy kittens can shed it in their feces, the sick kittens we hypothesize are the ones where not only their shedding it in the feces, but it is attaching to their intestinal epithelium, and causing the diarrhea and the disease. We also hypothesize that this dysbiosis of the intestinal enterococci, is what predisposes that gut to being vulnerable for those EPEC to adhere.

So where do we want to go with this? Obviously lots and lots of questions. Can EPEC that are attaching and causing diarrhea be distinguished from those who aren't? Can we take a cat and get EPEC from its feces, and ask something and test something about that EPEC, and determine that that EPEC is likely to be adhering inside that kitten and it is important, versus the one we might find in a healthy kitten? So maybe it is virulence gene expression, or serotype, or this DNA laddering, or presence of the ability to swim, or maybe it is some biomarker of the concurrent evidence of enterococcal dysbiosis, something that gives us another level of evidence for the importance of it in a particular kitten.



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Is EPEC a direct cause of diarrhea, or is it a co-conspirator. Is there something else going on in these kittens that if we can fix that, they wouldn't get disease from EPEC. So is it a current parasite or a virus, or another bacteria that is affecting the pathogenicity, or the role of the microbiome, the diet, stress, failure of the maternal derived immunity. So is there something else we need to look at? These things we are also looking at right now in these same kittens. Finally, can a probiotic, especially this *E. hirae*, prevent or ameliorate diarrhea caused by EPEC in kittens? Can we get kittens that probiotic, fix that dysbiosis, and prevent them from becoming colonized and sick by EPEC.

I greatly appreciate all of your attention. I am proud to be here, and I would not be able to do any of this without the Winn Feline Foundation. I also would say I wouldn't be able to do anything about this without all of the people on this slide. It is been an enormous effort, between a lot of labs, a lot of people with a lot of expertise that goes way beyond my own, and also in other universities like Kansas State University. So I appreciate your time.

END OF THIS AUDIO SECTION