Nikki Shariat: Good afternoon and thank you very much to the organizers for the invitation to speak at this exciting roundtable this afternoon.

Although I've worked in food safety for about a decade now it's only been in the last three years that I've been at the Poultry Diagnostic and Research Center that I've had the opportunity to work directly with integrators, concerning Salmonella in poultry production. And one of the messages that I've heard several times from different integrators is that the Salmonella serovars they identify in live production don't always match the serovars that they find in the processing plant. One of the typical scenarios is that they find serovar Infantis during processing, but when they go back and look at their environmental samples from preharvest, the serovars that they typically identifying are Kentucky.

So, the study I'm going to share with you today addresses why this might be happening. And this has been a wonderful collaboration with my research group at UGA, specifically with my PhD student Amy Siceloff with Dr. Doug Waltman at the Georgia Poultry Lab Network. And this is a project that's been funded by the USDA NIFA.

I have three approaches that we used in this study that I'll show you today. The first was to deeply analyze publicly available FSIS data over a five-year period, and then to compare this with processing establishment data with Salmonella monitoring data from breeder flocks in Georgia. And then we finished this with some multi-serovar population analyses in breeder flocks.

So, the first approach was to look at the serovars that are found in processing facilities. And so, FSIS collects this data and makes it publicly available. The pie charts that I'm showing here are looking at Salmonella incidence in carcass rinses and also in raw intact parts collected at processing. And the size of the circle reflects the number of Salmonella that were identified in that particular year. First, you can see that there is a reduction in Salmonella incidence in parts. They peaked at 16.8% in 2016 and although the number of Salmonella stays the same, the incidence is actually reduced to 7.8% in 2020, despite almost more than double the number of samples being collected. I've indicated some important serovars here on these pie charts. Each of these different colored slices of pie reflects a different serovar. And there are a few trends that we found. The first is the reduction in Heidelberg. Over the last decade Heidelberg has been a serovar of concern in poultry and you can see that there's been a nice reduction since 2016 to 2020 in this particular serovar. We also know that there is an increase in Salmonella Infantis that is associated with broiler production, and you can see that in both carcasses and parts the relative amount of serovar Infantis has increased. That's shown in the dark blue pie slice. By 2020, nearly a quarter of the serovars that were identified in parts were serovar Infantis. We also saw some interesting trends when we compared the serovars that FSIS identified in carcasses versus those in parts. And that is a reduction in Salmonella Kentucky. This is shown in red. You can see that in carcasses, about half the Salmonella that are identified are Kentucky and that is

dramatically reduced in intact parts sampling. Conversely, we see that the relative amount of Infantis stays the same, but that the amount of Enteritidis between carcasses and parts increases. Enteritidis is shown in this light green. You can see that it's consistently about a quarter of the Salmonella that's identified in raw parts are Enteritidis.

We then took this data and separated it by region. We broke down each region and have listed them here in order of the size of their production. The southeast is the largest broiler production in the US and they're shown at the top. The same four trends that I showed on the last slide hold true for each of these regions. But we also find some interesting regional differences. Proportionally, we find a higher amount of Typhimurium, shown in this light blue color, and Infantis in the Atlantic region compared to other regions in the US. And in the southeast, we see a very strong signal for serovar Schwarzengrund that's not observed in other regions.

When we look just at the Georgia data, the bottom two rows here are the FSIS data, specifically the state of Georgia over those same five years. We can see that the trends that I showed on the last two slides are very similar. We also included Salmonella serotype analyses from breeder samples. There's two sets of data in here. This is incidence data that Doug Waltman at GPLN has shared with us. Many integrators will monitor their breeder flocks at 16 weeks — that's before the birds go into production — and at 42 weeks. That's after the birds' peak production. And then hatching egg companies, in accordance with NPIP regulations, will monitor for Salmonella every 30 days. In this data here in these top two rows we have data from hatching egg companies and breeders. There's 14 different companies represented in total. The incidence for the top two rows has been divided by ten. Obviously we know we have a larger incidence of Salmonella in live production than we do in processing, but my pie charts would be too big to fit on this slide otherwise. But hopefully you can see that the take home from this slide is that proportionally, the amount of Salmonella Kentucky is much, much higher in breeders than it is at processing. Over three quarters of the samples at live production are accounted for by serovar Kentucky and this is significantly reduced in processing. We also see a slightly higher incidence of Typhimurium at this earlier time point compared to a later time point where we see an increase in Salmonella Enteritidis.

So, one of the hypotheses with this would be that perhaps Kentucky is being eradicated in the plants. And maybe Kentucky as a serovar is more sensitive to the interventions that you use during processing. Nonetheless, the serovars that we find in processing would have had to originate from live production. Perhaps in live production, they are being outcompeted by serovar Kentucky. So what I mean by this is that there is a limitation to the way that we currently perform Salmonella isolation. This is true of all diagnostic and regulatory facilities. When you have your Salmonella on a plate, you pick a small number of colonies — typically one to three colonies — and you characterize those. If in this case Salmonella Kentucky is the most prevalent serovar, statistically when you pick those colonies off the plate it would be the Salmonella Kentucky that you

identify and perhaps other serovars that are present in the background would remain hidden. My lab has developed a technology called CRISPR-SeroSeq, which is an amplicon-based sequencing approach to be able to profile several different serovars that may be present in a single sample to overcome this limitation.

I'll briefly show you how this approach works. So CRISPRs are genetic elements that are present in Salmonella. Salmonella has two different CRISPRs. And their CRISPR components genetically tracked very well with the serovar identity. I've shown these CRISPR sequences as these square blocks. If we know that in serovar A we have a specific pattern of CRISPRs. In serovar B we have a very distinct, separate set of CRISPRs. Our template for CRISPR-SeroSeq is typically an overnight culture of Salmonella. This might be a tetrathionate selective enrichment culture, for example. In the scenario I'm showing you here there are two different serovars that are present in this mixed population, serovar A and B. We perform a single PCR step to be able to amplify each of the different CRISPR elements that are present in that population. These are using primers that target the individual direct repeats for Salmonella. From that PCR we then tag those with Illumni barcodes so that we're able to multiplex those samples and sequence multiple samples at the same time. We typically use an Illumina platform. From our sequence reads we match these back to a database that we have in our lab that has 135 different serovars and we're able to say that serovar A and serovar B were present in this original population. We can also take the sequence read frequency for each of those spaces and use that information to calculate the relative frequency of those serovars to each other. So, in this situation, 95% of the Salmonella in that population belonged to serovar A. Five percent was serovar B.

So, this again was another collaboration with Doug Waltman at the Georgia Poultry Lab and he shared with us samples from enrichment cultures that are part of Salmonella monitoring. We looked at samples over a one-year period from July 2020 to June 2021. For this, we looked at samples that were collected on a different day each week so that we didn't have any bias with one specific company or integrator always submitting their samples on a Thursday, for example. And we looked, on average, at two to four Salmonella positive samples each week. So, for this study we have 134 samples that we've analyzed using CRISPR-SeroSeq. The different serovars that we found are here on the left-hand side. I've color coded some of the important serovars as per the pie charts that I showed earlier. You can see that in this particular sample, each column will be a single sample, we identified four different serovars: Enteritidis, Infantis, Kentucky, and Schwarzengrund. And the darker the color, the higher the relative frequency of that specific serovar in that sample, according to this key here.

So, this is just a snapshot of the data for one month. This is for the month of July 2020. You can see, unsurprisingly, and based on the surveillance data that Doug had already shared with us, that all eight samples that we collected in July contained Salmonella Kentucky. Half of these samples contained a single serovar, and each of these was serovar Kentucky. Kentucky was the major

serovar — so, the dark blue here — in six out of the eight samples. And half of the samples contained more than one serovar. In one sample here, we had four, three, three, and two different serovars.

Over a third of the samples that we examined over the year had more than one serovar. The range was from one serovar to eleven serovars. We found eleven serovars in one single sample that we collected in December. And we found 26 different serovars in total, five of which were in the CDC top ten. If I can draw your attention here as well to the serovars that are listed, perhaps it's easier to see on here – the serovars listed here on the side. You can see that some of them have multiple numbers. So here I've got Kentucky one and Kentucky two. And I've got Montevideo one, and Montevideo two, three, and four. So many Salmonella serovars are polyphyletic. So they have multiple different distinct genetic lineages. In the case of Kentucky, for example, this tracks very well with virulence. We know that Kentucky two is more commonly associated with human illness outside of the US and Kentucky one has a lower association with human illness and is the major Kentucky that we find in the United States. And so this shows that CIRSPR-SeroSeq can provide a slightly higher resolution than just serovar identity.

I summarized the data over this year by the serovars that were typically a major serovar or a minor serovar. And what I mean by that I've shown here. Here I've shown three different samples: A, B, and C. In A, Kentucky is the major serovar. Entrititids, Infantis, and Schwarzenbrund are the minor serovars. In sample B, Typhimurium is the major serovar, Kentucky and Entritidis are the minor serovars. And in sample C, Kentucky was the only serovar we found and it was the major serovar.

When we summarize all of this data, we see some interesting trends. So, for Salmonella Kentucky we identified this in 104 out of the 134 samples that we found. In terms of its incidence as the major serovar in a population versus the minor serovar. 93 out of those 104 times it was the major serovar and this corresponds to 89 percent of the time that we found Kentucky it was the major serovar, 11 percent of the time it was the minor serovar. What this means is that if you have Kentucky present in a sample, more often than not it's going to be the most abundant serovar and it will be easy to detect when picking colonies off of a plate.

When we look at Salmonella Infantis, you can see that we identified it in 12 samples and you can see almost the complete opposite effect. Eleven times out of those twelve, Infantis was a minority serovar. That corresponds to 92 percent of the time that we find Infantis it's in the background. We've since increased this study to look at over 340 different samples from breeders, and in that study we found Infantis 39 times, 34 of those times it was in the background. So this does seem to be a significant trend that we're finding in live production. What that means is that when Infantis is present in a sample it's typically going to be outnumbered, it's going to be hard to identify it when picking it off of a plate. And so in part we think that this explains that discrepancy between the live

production serotype incidence that Doug has shared with us and the FSIS plant data.

And so, to summarize, there are caveats to this project. These are very much trends that we've seen. We haven't done the study where we've sampled breeders, broilers, and processing from the same integrator. One of the goals of this project was to really see what was going on from a broad landscape. The missing piece of the puzzle are the broilers. We know from work from several groups, especially Chuck Hofacre and John Maurer, that the serovars we find in our breeders are typically those that we then finding broilers and we then find in processing, but it would be nice to be able to do this study by looking at broilers as well. We didn't use broilers, I didn't say that here, but there really isn't any kind of program, regionally or nationally, to monitor Salmonella serovars in broilers. And working with Doug, we were able, so many integrators will submit breeder samples for monitoring but they don't do the same for broilers. Many integrators do that analysis in house.

The other caveat was we see that multiple serovars do occur quite frequently. In a third of our samples we found multiple serovars present, but this analysis was only performed on breeders in Georgia. But in summary we can show that Salmonella serovars that we find in live production don't always match what we find in the plant. I think that the presence of multiserovar populations is one piece of the puzzle. Phenotypic differences among the different serovars is another piece of that puzzle.

In terms of moving forward, the data that I've shown here has implications for how and when we monitor for Salmonella in live production — when is the best time to sample, can we sample from broilers. And the goal would be that integratrors could use the information from these multi-serovar analyses to be able to select for appropriate serovars that they want to include in their autogenous vaccine and to perform complex biomapping to be able to identify different regions or different farms or flocks or possessions within a complex where some Salmonella serovars are entering the production chain.

With that I'll wrap up and say thank you to my lab, to Amy, and specifically to Doug who, without Doug's collaboration this project never would have happened so we're very much indebted to Doug. And thank you very much.