

***“Investigation into the effect of in-feed flavophospholipol on Salmonella shedding and antimicrobial resistance in pigs”***

Saranya Nair

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**Hannah Golightly:**

Good afternoon and thank you for joining us for the 5<sup>th</sup> presentation in a multi-part webinar series by the Ontario Swine Research Network. My name is Hannah Golightly and I am a second year veterinary student here at the Ontario Veterinary College who has been assisting with the OSRN activities.

The OSRN has been formed by faculty at the University of Guelph and representatives from Ontario Pork, OMAFRA, and the swine veterinary community. The goal of the network is to enhance and improve the timeliness and accuracy of U of G swine research results and activities to end users. We also aim to highlight the ongoing collaborative work taking place with other institutions and research partners in order to capture the provincial, national and international impact of the U of G's Swine Research Program.

We have launched a website, [www.uoguelph.ca/osrn](http://www.uoguelph.ca/osrn). It's a provider platform where producers, veterinarians, industry, students and others can go for current and archived research results. Before I introduce our speaker, I would like to inform you of some of the features of our webinar platform, Adobe Connect. You may enter a comment under the chat window, which should be at the bottom right hand of your screen. To keep the webinar flowing, we will take questions at the end of the presentation.

This afternoon I am pleased to introduce today's speaker, Saranya Nair. Saranya is a first-year PhD student in the Department of Population Medicine under the supervision of Dr. Robert Fletcher. Prior to starting her PhD, she completed her BSc in Life Sciences at McMaster University and her MSc in the Department of Population Medicine at the University of Guelph. Her research interests include zoonotic diseases, antimicrobial resistance, and public health.

With that, I will turn it over to Saranya to take you through her presentation entitled “Investigation into the effect of in-feed flavophospholipol on Salmonella shedding and antimicrobial resistance in pigs”.

**Saranya Nair:**

Thank you, Hannah. I would just like to start by thanking Hannah and Terri for inviting me to do this webinar today. So I'm going to be presenting on my master's work, the investigation into the effect on in-feed flavophospholipol on Salmonella shedding and antimicrobial resistance in pigs. So, for the duration of the presentation I will be referring to flavophospholipol by its trademark name, Flavomycin.

So the aim of this talk will be to answer two questions, so our main question being: Does Flavomycin help to reduce Salmonella shedding and antimicrobial resistance in pigs? And then our secondary question, which is quite interesting and that I would like to add to this presentation is: What do we find in this population of pigs that we worked with for this trial? And this pertains to the problems of Salmonella serotypes and seroprevalence.

So the emergence of multi-antimicrobial resistant Salmonella in swine is a concern to food safety and public health. Salmonella is commonly found in the faeces of pigs. It can be recovered from 70% of Ontario swine farms with close to market pigs. Research has also suggested that it can impact pig performance, and so the basic trend of Salmonella in pigs is that they tend to shed the most during the nursery stage, and from there there's a decline up until slaughter. A lot of the times pigs are asymptomatic carriers – they're infected but they don't shed Salmonella. However, in extremely stressful situations like transport to slaughterhouses or waiting in high-capacity holding areas prior to slaughter, the asymptomatic carriers start shedding Salmonella, and that's where the asymptomatic pig plays a significant role in the spread and transmission of Salmonella and associated antimicrobial resistance from farm to fork.

Another important route of transmission is through cross-contamination of produce, crops, and ground water when Salmonella-infected swine manure is used as a land fertilizer. So there is a lot of control of disease transmission that happens at the slaughterhouse. However, one of the best ways to control and minimize the transmission of Salmonella and associated antimicrobial resistance to humans via food products is to control Salmonella at the farm level.

So, how can we control Salmonella shedding and the associated antimicrobial resistance? Well, there are various control measures like cleaning, disinfection, vaccinations, et cetera. However, these methods alone, or combined, have a low response in controlling Salmonella in pigs. Previous studies have shown promising results with the use of Flavomycin, an antibiotic produced by streptomyces strains as it may potentially reduce Salmonella and associated antimicrobial resistance. Due to its four pharmacokinetic and pharmacodynamic features. Flavomycin does not meet the minimum requirement to be classified as a therapeutic antimicrobial in human and veterinary medicine. The antibiotic develops resistance slowly through chromosomal mutations making it non-transferrable and safe to use in animals, and so it's been used for over the past thirty years primarily as a growth promoter in livestock.

Furthermore, it's also believed that Flavomycin may have a plasmid curing effect resulting in the reduction of antimicrobial resistance, but inhibiting the growth of bacteria like Salmonella and E. coli that contain R plasmids. So research has suggested that Flavomycin by altering the gut microflora in favour of beneficial bacteria can possibly inhibit colonization of Salmonella in swine, while potentially having a positive impact on the growth of pigs, which is a great economical incentive for swine producers to use Flavomycin.

So, based on this information, we decided to investigate whether in-feed Flavomycin can produce Salmonella shedding and antimicrobial resistance associated with Salmonella and E. coli in grower-finisher pigs. And the reason that we decided to look at generic E. coli as well was in case the pigs were not shedding Salmonella. And we know that generic E. coli is present in the gut of pigs, and there is also literature suggesting that Flavomycin may be effective in reducing antimicrobial resistance in E. coli. We also looked at Salmonella shedding pattern and Salmonella colonization in these naturally infected pigs through the grower-finisher stage and at slaughter, as well as the development of antibody in relation to Salmonella shedding.

So, in order to meet these objectives, we used 45 naturally infected pigs at 10 weeks of age, weighing approximately 35 kg. These pigs were purchased from a commercial farm with a history of Salmonella, and prior to the start of the study we tested the sows and the piglets on the farm and they demonstrated a high prevalence of Salmonella.

The pigs were housed at Ponsonby General Animal Facility here at the University of Guelph into 9 pens with 5 pigs per pen. 5 pens of 25 pigs received 4 ppm of Flavomycin in-feed with the remaining 20 pigs in 4 pens receiving a non-medicated control. So initially we had one room with 9 pens – 5 treatment and 4 controlled – but due to the lack of room in pens as pigs grew from week 8 until the end of the trial, we moved 2 to 3 pigs from room 1 to room 2 into the same treatment or a controlled pen. So room 2 mimicked the setup of the initial room. So from week 8 until the end of the trial we had 18 pens in total – 10 treatment and 8 controlled.

So over the 10 weeks the clinical trial with Flavomycin was conducted individual fecal samples were collected weekly. Fecal samples collected at week 1 were before the pigs started receiving treatment. Blood samples were collected at week 1, 4, 7 and 11, and 2 to 3 weeks following the end of trial pigs were slaughtered at the University of Guelph abattoir were tissue samples – so ileocecal lymph node, neck lymph node, spleen, liver, tonsils and cecal contents were collected from each pig.

So in terms of the laboratory work, I won't be going into much detail, but the takeaway from this slide is that we used the collected fecal and tissue samples to culture for Salmonella from which we serogrouped and serotyped the first and the last Salmonella isolates for positive culture for a pig, and we also used the fecal samples to culture for the generic E. coli, which we used week 1 and week 11 samples.

For antimicrobial susceptibility testing, pigs with Salmonella-positive isolates at week 1 before treatment, and week 3 and 4 after treatment, were used while E. coli isolates from pigs at week 1 and 11 were tested. So using the Broth Microdilution Method on a NORMS Gram negative plate, we used a Sensititer System to read the plates.

Blood samples were centrifuged and the serum was extracted and using ELISA method the level of antibodies in pigs was measured, and the optical density

values found using ELISA were adjusted using the equation instructed by the manufacturer, and from there were classified as seropositive or seronegative.

So in this study we had clustering at two levels due to common environment at the pen level and due to repeated measure at the pig level. So random effects was used to control for this. Furthermore, the addition of a second room was controlled for using mixed-effects. So based on this, multilevel mixed-effects, a logistic regression model was used to compare Salmonella shedding between the treatment and the control groups, antimicrobial resistance between the treatment and the control groups, prevalence of Salmonella shedding over time as pigs age, and lastly, to evaluate the association between antibody response (seropositivity) and Salmonella shedding.

So the results revealed that over the 10 weeks of sample collection, all pigs tested positive for Salmonella shedding at least once, and 89% of the pigs more than once. The overall mean prevalence of Salmonella shedding was 27% from week 1 to week 11, and so here you can see that Salmonella was recovered from 80% of the pigs at arrival to the research facility, and from there you can see there's a decline in week 2 to 38% prevalence, and that it peaks to 91% at week 3. And then from week 3 onwards there is a decreasing trend in Salmonella shedding.

Salmonella shedding was assessed over time as pigs age, and so the ordinal data revealed that as pigs age from week 1 to 3 ... week 1 to week 3, so this is 11 weeks of age to 12 weeks of age, that there was a significant increase in Salmonella shedding in comparison to week 1 to week 2. However, from week 4 ... sorry, from week 3 to week 11, so this is 12 weeks of age to 20 weeks of age, there is a significant decrease in Salmonella shedding in comparison to week 2 to week 3. However, overall, we found that there was a decrease in Salmonella shedding, but the possibilities behind the variation in Salmonella shedding in the early growth stage will be further explained later in the presentation.

So this population of pigs were repeatedly positive throughout the grower finisher stage and at slaughter. So here we have a chart that groups a pig by how many times they were Salmonella positive by the trend of shedding. So the dark red columns identify Salmonella positives and here at the top we have week 1, W1, up until W11, which is week 11, and then the last column SH, that is slaughter.

So we found out that out of the 45 pigs, 41 pigs were positive 4 times or less, and 4 pigs tested positive 5 times or more, and we classified these pigs as our chronic shedders. We also had one pig that was positive 9 times over the duration of the trial and at slaughter.

In terms of slaughter, we recovered Salmonella in the liver, the spleen, neck lymph node, ileocecal lymph node, tonsils and cecal content. So out of the 43 pigs that survived to slaughter, 7 of them were Salmonella positive at slaughter, with one pig being positive in 2 tissue samples. And, interestingly, out of the 7 tissue positive pigs, we had 5 pigs who were negative for 7 to 8 weeks prior to

slaughter, and then at slaughter we found Salmonella in the lymph nodes, tonsils and cecal content.

And so here you can see there is a 7- to 8-week gap. They stopped shedding at week 3 and week 4, so at 12 and 13 weeks of age, and so these are asymptomatic pigs that were no longer showing clinical signs of Salmonella, however are positive in tissue samples. So this could be a concern for food safety because sometimes lymph nodes do make their way into processed meats, and that could potentially lead to a large outbreak of Salmonella.

In terms of serogrouping, we found isolates belong to serogroup B, C1, E4 and rough, with the greatest number of isolates 44% serogrouped for C1. In terms of the slaughter isolates, they belong to serogroup C1 and group B, and interestingly 70% of the pigs that were serogrouped twice, the first and last isolates, had a different serogroup. So here we have the first isolate and the last isolate, and then the number of pigs that had a change. The greatest change was seen from serogroup E4 to C1, followed by no change.

It's also important to mention that pigs with Salmonella belonging to serogroup C1 had no change in serogroup. So this is interesting because certain serogroups may have cross-protection, so protecting them from being infected with another serogroup, and this would be something to look into in future research. It's also worth mentioning that the majority of the changes in serogroups was during week 3 and 4. From there the serotyping data revealed that there was multiple serotypes present in the small population of pigs. A total of 8 serotypes, with the most prevalent being *S. Typhimurium* followed by *S. Livingstone*, and at slaughter *S. Typhimurium* and *S. Infantis* was commonly recovered.

And so, of the 11 pigs that were serotyped for first and last isolates, 10 of those were re-infected with different serotypes. And so here we have the first and the last isolates of colonized pigs, and from this you can see the most common re-infection was by *Livingstone*. And so, based on this information, when we go back to the Salmonella shedding over time, variability in Salmonella prevalence and shedding pattern in the early grower phase is probably a result of the reinfection caused by the presence of multiple serotypes.

From there, when we go over to evaluating the association between Salmonella shedding and antibody response, we found that the predictive probability of Salmonella shedding decreased as the level of antibodies increased. So, if we were to look further into Salmonella infection and host antibody response, we see that as the mean adjusted OD level – so the antibody response – increases, the Salmonella shedding is decreasing in pace.

And so, furthermore, the multilevel mixed effects logistic progression analysis revealed that Salmonella-shedder pigs are less likely to be tested seropositive by ELISA than non-shedder pigs, which is what we saw here. When the pigs are bacteriology positive they are serology-negative. And as time progresses we see that they become bacteriology negative as they become serology-positive. In addition, we found that the peak in Salmonella shedding in bacterial culture was at 12 weeks of age, whereas the peak in seroprevalence is at 16

weeks of age, so there is a 4 week, 28 day lag between the peak in Salmonella shedding in the early grower finisher stage and the peak in host antibody response in mid-finishing.

So we saw that there was a low level of Salmonella shedding towards the end of the trial, but we found 7 pigs positive at slaughter. However, we also found a high mean level of antibody in pigs at week 11. So could we use serology to detect intermittent shedders? Well, when I went back and assessed the antibody levels in pigs that were Salmonella positive at slaughter I found only two pigs were seronegative, one at week 11 and the other pig was seronegative during the entire trial, whereas 5 of the other pigs were seropositive. So there is a possibility that by using serological testing methods we may be able to identify pigs that are colonized by Salmonella when bacteriology culture is not able to identify the asymptomatic Salmonella carrier pigs.

So now that we have an understanding of the Salmonella infection, the multiple serotypes and the antibody response in these pigs, I would like to focus the remainder of the presentation to Flavomycin. So the findings revealed that the controlled group had a mean Salmonella prevalence of 28%, whereas the treatment group had a mean prevalence of 27%. And so you can see from this graph both the treatment and the controlled groups had a very similar pattern of Salmonella shedding over the 10 weeks. And so statistical analysis revealed that there was no difference in Salmonella shedding between the Flavomycin treated pigs and the non-medicated controlled pigs.

And so the next question was, why did it not work? Well, as I had mentioned earlier, there was variability in Salmonella shedding in the early grower stage due to reinfection caused by the presence of multiple serotypes. Previous challenge studies that showed Flavomycin effectively reducing Salmonella used pigs that were infected with one serotype, whereas in this study we have a population of pigs infected with multiple serotypes, so this makes it difficult to measure the effectiveness of Flavomycin.

Also, there is a possibility of residual infection from the nursery that may have lingered into the grower finisher stage. If a high degree of Salmonella colonization occurred in the gut flora prior to the pigs receiving treatment, this may have enabled Flavomycin from having an impact on the beneficial bacteria. So if these same pigs were provided with a treatment diet at an earlier stage, perhaps after weaning when the pigs were younger and most susceptible, a more favourable outcome may have occurred. And so future studies examining the benefit of administering Flavomycin as a preventative measure before pigs are infected with Salmonella needs to be investigated.

In terms of antimicrobial resistance in Salmonella, from week 1 to week 3 the pigs demonstrated resistance to Streptomycin, Sulfisoxazole, Tetracycline, Ampicillin and Chloramphenicol. And although in this graph you see that there is a greater resistance in the treatment group than the control group, the change in resistance from week 1 to week 3 between the control and the treatment groups had no difference. Similarly, from week 1 to week 4 the pigs also demonstrated resistance to the Streptomycin, Sulfisoxazole, Ampicillin, Tetracycline and Chloramphenicol, and again the change in resistance from

week 1 to week 4 between the control and the treatment groups had no difference. And so Flavomycin treated pigs and the controlled pigs had the same change in antimicrobial resistance.

So at this point findings revealed that Flavomycin was not able to reduce Salmonella shedding and associated antimicrobial resistance in pigs. However, when we analyzed the resistance in the E. coli isolates we found resistance in multiple antibiotics, but there was a significant lower change in a difference in Ampicillin, Chloramphenicol and Trimethoprim/Sulfamethoxazole resistance in Flavomycin treated pigs. So these findings support previous challenge studies that found Flavomycin to reduce antimicrobial resistance. Another point worth mentioning is that the Salmonella isolates were analyzed from week 1 to week 3, and week 1 to week 4, due to a large prevalence of Salmonella during those weeks. Whereas the generic E. coli isolates were compared from week 1 to week 11. So there is a possibility that more favourable results were not seen in Salmonella isolates because we measured the change in resistance after 2 and 3 weeks of treatment.

So overall, although this small population of pigs were from a single source, had a high level of Salmonella shedding, and multiple serotypes, they were fairly healthy-looking pigs. If we had visited these pigs on a commercial farm close to market and saw a low prevalence of Salmonella, we may have concluded that the herd was negative, and we have seen various studies like that. And that's what makes this study we conducted fairly unique in that we followed the same pigs for such a long period of time up until slaughter, so we were really able to see how long pigs shed Salmonella. Furthermore, these pigs are representative of a highly Salmonella-infected farm and we were able to see whether Flavomycin was able to control Salmonella or not.

So, in conclusion, the take-home messages from this research is that Salmonella infection peaked in the early grower stage and declined as pigs aged. There was a 4 week lag between the peak in Salmonella shedding to the peak of host antibody response in mid-finishing stage. ELISA may be used to identify the carrier pigs that might have been colonized. Absence of Salmonella detection in fecal samples in late finisher stage isn't an indication that Salmonella will not be found in tissue at the time of slaughter posing a food safety concern. Flavomycin used as a control method was not effective in reducing Salmonella shedding or antimicrobial resistance in this study, while E. coli isolates from pigs treated with Flavomycin had reduced resistance in comparison to controlled pigs.

In terms of future direction, it will be beneficial to assess the preventative measures of Flavomycin on Salmonella and antimicrobial resistance at an earlier age of production, like in the nursery pigs. Evaluate the most efficient dosage of Flavomycin for reducing Salmonella shedding and antimicrobial resistance. Explore cross-protection among serotypes, and further research into serological testing to identify asymptomatic Salmonella-carriers.

So now if we were to go back to the agenda slide, our main question being: Does Flavomycin help to reduce Salmonella shedding and antimicrobial

resistance in pigs? Do we know this? Not really. Although this study showed that it wasn't very effective, I think further research needs to be conducted to determine this. The secondary question: What did we find in this population of pigs? We found a high prevalence of Salmonella, multiple serotypes, reinfection, and colonization in tissue and lymph nodes.

And so with that I would like to conclude my presentation and thank my committee, Dr. Robert Friendship, Dr. Vahab Farzan, Dr. Terri O'Sullivan, Jane Newman, and the Pig Group, Ponsonby, and CPHAZ for all their help and assistance with my master's project, as well as our funders. And so with that I would be happy to take any questions. And so, I guess in that little bit of time that we have while questions are coming in, I can just answer some commonly asked questions.

So something that usually comes up is people tend to ask: Do you think that providing the pigs with a greater dosage of Flavomycin in-feed would have provided better results? And I ... I think so, it might have. So a lot of the challenge studies that have reported Flavomycin to be effective have found that providing the pigs with a dosage- I think one of the studies was 9 parts per million, that they found that to be very effective. So we provided these pigs with 4 parts per million in-feed, which is what is recommended on the label, and we found that not to be very effective. So maybe providing them with a greater dosage may have worked better, especially because these pigs had such a high prevalence of Salmonella. So that would be something to consider in the future.

Another question would be: Would we have seen better results in terms of antimicrobial resistance if Flavomycin was assessed over a longer duration? And that is a possibility. We only looked at it over 2 to 3 weeks in terms of the Salmonella isolates, whereas in the E. coli isolates we looked at it over 10 weeks. So if we had assessed antimicrobial resistance over a longer period of time we may have seen better results. And some of the studies that have reported positive results have been over a longer duration. But then again, we had so many different stereotypes it could vary; a lot of the challenge studies used Salmonella typhimurium.

And my last question that I get a lot is: How does Flavomycin impact a microbiome? And that is what I am currently working on for my PhD and we're looking at how Flavomycin could impact the microbiome versus what happens in controlled pigs when they're infected with Salmonella typhimurium. Okay, so I'm going to pass it over to Hannah.

### **Hannah Golightly:**

Thank you for that great presentation, Saranya, I look forward to hearing the results of your microbiome work in the future. And thank you to everyone for joining us today. Remember to visit our website, [www.uoguelph.ca/osrn](http://www.uoguelph.ca/osrn), for recordings of more presentations and other swine research resources. Have a great weekend, everyone.