Title: Phage therapy to reduce transport and lairage associated increases in *Salmonella* infections: evaluation of delivery methods - **NPB #09-131**

Investigator: Paul Ebner

Institution: Purdue University

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Industry Summary

Bacteriophages are naturally occurring microorganisms that target and destroy bacteria. Our laboratory previously demonstrated that administering bacteriophages to pigs reduces *Salmonella* colonization when those pigs are exposed to a contaminated environment. These results indicated that bacteriophages could be useful in controlling increases in *Salmonella* shedding that occur just prior to processing due to transportation and lairage. The purpose of the current project was to increase the efficacy of our phage treatment by: 1) increasing the host-range to include other common *Salmonella* serovars associated with swine; 2) developing a more affordable microencapsulation process to protect the phages from stomach acids; and 3) making deliver more practical by determining whether phages can be direct fed. We collected samples from wastewater treatment facilities and isolated 20 distinct phages belonging to either the Siphoviridae or Myoviridae families. From this library we identified 10 phages that together lysed a mixed culture of *Salmonella enterica* Typhimurium, Enteriditis, and Kentucky. Phages were microencapsulated using two methods that had minimal effect on phage viability. Microencapsulated phages remained stable at various temperatures for up to 14 days with no appreciable drop in viability. Twenty-one pigs in three replicates were randomly placed into three groups: feed (F), gavage (G), and control (G). The F group was direct fed the phage cocktail daily for five days. On the fifth day, the G group received the same phage cocktail by gavage, while C pigs received a mock treatment with no phage. All pigs were then challenged with *Salmonella enterica* Typhimurium. Pigs in the F group were significantly less likely to shed *Salmonella* post-challenge compared to pigs in both the G and C groups. Likewise, the concentrations of *Salmonella* in the ileal and cecal samples were significantly lower than ileal and cecal samples from C pigs. Taken together, these results indicate that direct feeding phages is a practical and effective means of reducing *Salmonella* colonization and shedding in pigs.

Keywords

Salmonella, phage, food safety, transportation, lairage

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For more information contact:

National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

Scientific Abstract

We previously produced an anti-*Salmonella* phage cocktail that reduced colonization in swine when the pigs were exposed to an environment heavily contaminated with *Salmonella*,
similar to what might be seen in a transport trailer or processing facility holding pen. In this study we increase the efficacy of the phage treatment by (1) expanding the host-range of the cocktail; (2) developing a more cost-effective microencapsulation technique; (3) improving the delivery method. We collected samples from wastewater treatment facilities and isolated 20 distinct phages belonging to either the Siphoviridae or Myoviridae families and identified 10 phages that together lysed a mixed culture of *Salmonella* enterica Typhimurium, Enteriditis, and Kentucky. The phages were microencapsulated using two sodium-alginate-based methods that only reduced the cocktail titer by 1.0–1.5 logs (premicroencapsulation: 10.4 log10 PFU/mL; post-microencapsulation method one: 9.2 log10 PFU/mL; post-microencapsulation method two: 8.9 log10 PFU/mL). Microencapsulated phages remained stable at both 4ºC and 22ºC for up to 14 days with no appreciable drop in titer (mean titer: 8.9 log10 PFU/mL). Twenty-one pigs (~35 lbs) in three replicates were randomly placed into three groups: feed (F), gavage (G), and control (G). The F group was direct fed a 14 phage cocktail daily for five days (1.2×10^{11} PFU/day). On the fifth day, the G group received the same phage cocktail by gavage (1.2×10^{11} PFU), while C pigs received a mock treatment with no phage. All pigs were then challenged with 5×10^{8} CFU of *Salmonella enterica* Typhimurium. At six hours post-challenge, all pigs were euthanized and ileal and cecal samples and mesenteric lymph nodes were collected and analyzed for the challenge organism. Pigs in the F group were significantly less likely to shed *Salmonella* at 2h (38.1%) and 4h (42.9%) post-challenge compared to pigs in both the G (2h: 71.4%; 4h: 81.1%) and C (2h: 71.4%; 4h: 85.7%) groups ($P < 0.05$). Likewise, the concentrations of *Salmonella* in the ileal (2.0 log_{10} CFU/mL) and cecal (2.7 log_{10} CFU/mL) samples were significantly lower than ileal (3.0 log_{10} CFU/mL) and cecal (3.7 log_{10} CFU/mL) samples from C pigs. Taken together, these data indicate that direct feeding phages is a practical and effective means of reducing *Salmonella* colonization and shedding in pigs.

Introduction

The Centers for Disease Control report 40,000 cases of salmonellosis, including 400 deaths, annually in the United States (CDC, 2008). Roughly 95% of *Salmonella* infections are foodborne and most result from the consumption of contaminated meat, poultry products, and dairy products (Mead et al., 1999). It follows that there is an association between pork products and salmonellosis as swine are generally considered to be the second largest reservoir of *Salmonella* among food animals after poultry. Although infections in adult pigs are normally asymptomatic, once infected, swine can shed the organism in the feces for weeks and sometimes months (Ebner and Mathew, 2000).

While a great deal of research has been devoted to the development of on-farm *Salmonella* intervention strategies such as prebiotics, probiotics and other feed additives, each of these methods is confounded by the fact that pigs with no history of on-farm infection often arrive at the processing facility shedding *Salmonella* in high quantities. Previously, such increases in infections were attributed to transport stress and reactivation of already existing infections. More recent studies indicate that increases in *Salmonella* shedding may also be the result of new infections that the pigs obtain from contaminated transport trailers and/or holding pens (Larsen et al., 2004; Hurd et al., 2002 Rostagno et al., 2003). As such, it appears that on-farm efforts to reduce *Salmonella* loads in pigs must be complemented by intervention strategies that also prepare the animals for the contaminated environments that they will encounter immediately upon leaving the farm. Towards this end, we have developed an anti-*Salmonella* phage cocktail to combat transport and lairage-associated *Salmonella* infections. In previous experiments, we demonstrated that treating naïve pigs with a microencapsulated anti-

Salmonella phage cocktail prior to their comingling with *Salmonella* infected pigs in a contaminated pen significantly reduced new infections (Wall et al., 2008).

In those experiments, we administered the phage to the pigs orally by gavage. In this proposal we are seeking funding to develop a more practical means of delivery. We have shown that we can successfully microencapsulate the phage cocktail and we are confident that the treatment can be administered effectively via watering systems in its microencapsulated form. We also seek funding to increase our phage library. The increase would allow us to mix, match and rotate the actual phage strains in the phage cocktail which would increase the effectiveness of the treatment by circumventing any problems with resistance development, thus ensuring the success of the therapy over time. As our previous results demonstrate, we have been very successful in isolating and characterizing anti-*Salmonella* phage from various environments.

Taken together, these experiments represent the culmination of the development of an effective, practical and safe anti-*Salmonella* intervention strategy that producers or processors will very soon be able to employ to combat transport and lairage associated *Salmonella* infections in pigs.

Objectives

In previous experiments, we demonstrated that treating pigs with an anti-*Salmonella* phage cocktail prior to their introduction to a *Salmonella* contaminated environment significantly reduced new infections. Here we intend to build on these positive results by: 1) developing a means by which the phage cocktail can more practically be administered to large quantities of pigs through watering systems: and 2) increasing the size and diversity of our phage library to ensure that the treatment stays effective over time.

Materials & Methods

Bacterial and Phage Strains. *Salmonella enterica* serovar Typhimurium γ4232 was used for the challenge experiments. This strain was originally isolated from diseased pigs and contains a nalidixic acid resistance marker (Ebner and Mathew 2000). The phage cocktail contained 14 wild-type phages that were isolated from 14 wastewater treatments as previously described (Zhang et al., 2010; Wall et al., 2010).

Host-range analysis. Individual phages were tested for their ability to lyse *Salmonella* serovars Typhimurium, Kentucky, and Enteriditis. Each strain was grown to log phase and combined with the individual 14 phages. The coculture was incubated overnight and individual phage contrations were measured. In separate experiments, the phages were combined together (based on volume) into a cocktail and tested for their ability to lyse a mixed culture of the three *Salmonella* serovars together. Standard plaque assays were conducted using cocktail and a combination (based on volume) of *Salmonella* serovars Typhimurium, Kentucky, and Enteriditis.

Characterization of phages. Phage and microsphere morphology were determined by electron and light microscopy, respectively.

Microencapsulation. Phages were then combined into a cocktail based on volume and combined with 1.5% sodium alginate, Span-85, and canola oil. A 0.5% $CaCl_2$ / 0.05% $ZnCl_2$ was added and the solution was mixed and centrifuged. The supernatant was decanted and the pellet was resuspended in sterilized water. Microspheres were washed again, suspended in PBS and stored at 4ºC until further use.

Live Animal Trials. All animal experiments were conducted under the approval of the Purdue University Animal Care and Use Committee. Twenty-one small pigs (30lbs to 40lbs) in three replicates were randomly separated into three treatment groups. All pigs were screened for prior infection with *Salmonella* through fecal sample collection. One group (Feed) was administered the microencapsulated phage cocktail via feed daily for

five days. On average each pig received $1.2x10^{11}$ PFU of phage per day. On day five, a second group (Oral) of seven pigs received $1.2x10^{11}$ PFU of phage orally by gavage every 2h over a 6h period. A control group of seven pigs received no phage throughout the experimental period. On the fifth day all of the pigs were challenged with 5mL of 10^9 *Salmonella enterica* Typhimurium. γ4232. Fecal samples were taken every two hours for 6 hrs (0hr, 2hr, 4hr, 6hr). At six hours post-challenged, all of the pigs were euthanized and the contents of the cecum and ileum were gathered along with mesenteric lymph nodes.

Sample Processing. All media contained 50µg/ml of the nalidixic acid unless otherwise stated. Fecal swabs were enriched in 9ml of tetrathionate broth (Becton, Dickinson and Company, Sparks, MD) overnight in a 37ºC. Ileal and cecal samples were serially diluted and then directly plated onto XLT4 agar (Becton, Dickinson and Company, Sparks, MD) plates and incubated overnight. All samples were also enriched by adding 100uL to 9ml of tetrathionate broth and incubated overnight. The enriched culture (100µL) was added to 5mL of Rappaport-Vassiliadis Becton, Dickinson and Company, Sparks, MD) and incubated overnight at 42ºC. The double enriched sample (100µL) was then plated on XLT4 agar base agar plates and incubated in the 37C overnight. Lymph nodes were pulverized and placed in stomacher bags containing 50mL of tetrathionate broth and incubated at 37ºC overnight. Enriched cultures (100µL) were added to 5ml of Rappaport-Vassiliadis broth and incubated overnight at 42ºC. Double enriched samples were plated on XLT4 agar plates and incubated overnight at 37ºC.

Statistical Analysis. All bacterial concentrations were converted to a logarithmic scale before the data were statistically analyzed. The experimental unit was used was the individual pig. Significance levels were based on a P level of 0.05 or 0.1.

Results

Objective 1. Increase the host range of phage-cocktail.

Twenty phages were isolated from wastewater samples obtained from 14 different wastewater treatment plants throughout Indiana. These phages were isolated initially based on their ability to lyse *S. enterica* Typhimurium. Each individual phage was then screened for its ability to lyse *S.enterica* serovars Kentucky and Enteriditis as well. From the initial group of 20, 10 phages from 10 individual wastewater treatment plants were identified that lysed the different serovars. All phages lysed *S. enterica* Typhimurium (titer = 7.2 to 10.8 log10 PFU/mL). Nine of the 10 phages lysed *S.* enterica Enteriditis (titer = 6.1 to 10.8 log10 PFU/mL). Five of the 10 phages lysed *S.* enterica Kentucky (5 to 9.0 log10 PFU/mL) When combined together, the cocktail produced titers in excess of 10 log10 PFU/mL on mixed culture of *Salmonella* Typhimurium, Kentucky, and Enteriditis.

Objective 2. Develop a more affordable microencapsulation technique

Two microencapsulation protocols were tested. With both protocols, the 10 phages were combined into a cocktail before microencapsulation. The effects of the different microencapsulation methods on phage viability were tested by coculturing the microencapsulated phage cocktails with a mixed culture of the three *Salmonella* serovars. Nontreated (non-microencapsulated) phage cocktails produced titers averaging 10.4 log10 PFU/mL. Microencapsulation with method one reduced the phage concentrations to 9.2 log10 PFU/mL. Similarly, microencapsulation with method two reduced phage concentrations to 8.9 log10 PFU/mL.

Objective Three: Improve delivery mechanisms

In three replicates, 21 (3-4 week-old) pigs were randomly separated into three groups of seven pigs. One group received the anti-*Salmonella* phage cocktail in the feed (feed; $1.2 X 10^{11}$ PFU/day) for five days. On the fifth day, the second group received the same anti-*Salmonella* phage cocktail by gavage (oral; $1.2 X 10^{11}$

PFU/day) while the third group received a mock treatment (control). All pigs were then challenged with *Salmonella enterica* Typhimurium.

Fecal samples were collected from all pigs at 0hr, 2hr, 4hr and 6hr post-challenge. All fecal samples from pigs in all groups were negative for the challenge organism at 0hr. By 2hr post challenge, the challenge organism was detected in 71.4% of pigs in both the oral and control groups. A significantly lower percentage of pigs (38.1%; $P < 0.05$) in the feed group shed the challenge organism at 2hr compared to both the oral group and the control group. At 4hr post-challenge, 42.9% of pigs in the feed group shed the challenge organism compared to 81.1% and 85.7% in the oral and control groups, respectively. By 6hr post-challenge, 100.0% of pigs in all three groups shed the challenge organism in the feces.

All pigs were euthanized at 6hr post-challenge and mesenteric lymph nodes, ileal samples and cecal samples were collected. The concentration of the challenge organism was determined in both ileal and cecal samples. *Salmonella* concentrations in ileal samples from both the feed group (2.0 $\log_{10}$ CFU/mL) and the oral group (1.0 $\log_{10}$ CFU/mL) were significantly lower than *Salmonella* concentrations in ileal samples from control pigs (3.0 $\log_{10}$ CFU/mL; $P < 0.05$). Significantly higher percentages of pigs in both the feed group (42.8%) and the oral group (71.4%) had no detectable *Salmonella* (detection limit: 100 CFU/mL) in the ileum compared to pigs in the control group (19.0%; $P < 0.05$).

Salmonella concentrations in cecal samples from the feed group (2.7 $\log_{10}$ CFU/mL) were significantly lower than *Salmonella* concentrations in cecal samples from control pigs (3.7 $\log_{10}$ CFU/mL; $P < 0.1$). There was no significant difference between the *Salmonella* concentrations of the oral group (3.3 $\log_{10}$ CFU/mL) with the *Salmonella* concentrations of the feed or control group. There were higher percentages of pigs in both the feed group (28.5%) and the oral group (14.2%) had no detectable *Salmonella* (detection limit: 100 CFU/mL) in the ileum compared to pigs in the control group (9.5%; $P > 0.05$) but they were not significant. There was also no significant difference with the lymph node samples due to all being positive for *Salmonella* within each treatment group.

The cecal and ileal contents were analyzed for concentrations of the anti-*Salmonella* phage cocktail within each tissue for each pig. Concentrations of 1.4×10^6 PFU/ml and 8.5×10^6 PFU/ml were detected in the ileum and cecum, respectively, of pigs in the feed group. Concentrations of 2.0×10^4 PFU/ml and 2.2×10^3 PFU/ml were detected in the ileum and cecum, respectively, of pigs in the oral group. Concentrations of 2.2×10^3 PFU/ml and 3.8×10^3 PFU/ml were detected in the ileum and cecum, respectively, of pigs in the control group.

Discussion

It is well documented that *Salmonella* shedding in pigs often increases after transport and lairage. Producers and packers are very limited as to intervention strategies available to reduce *Salmonella* colonization during this period due. We identified bacteriophages as a potentially effective and practical way to combat transport and lairage associated increases in shedding. Bacteriophages have the advantage of being naturally occurring microorganisms with no known toxicity that specifically target bacteria. They are easily isolated from various environments and several groups have demonstrated that bacteriophages can cause significant short-term reductions in bacterial infections.

Our previous NPB-funded studies demonstrated that a cocktail of anti-*Salmonella* phages, when admistered orally (gavage), could reduce *Salmonella* colonization when treated pigs were exposed to a *Salmonella* contaminated environment. Treating individual pigs by gavage prior to transport, however, is impractical. In the present study, we demonstrated that a new, more affordable microencapsulation technique protects phage viability for up to 14 days at various temperatures, indicating that the treatment could be administered to pigs in the feed. In live animal trials, direct feeding phages significantly reduced *Salmonella* colonization compared to mock-treated pigs. These results demonstrate that bacteriophage therapy can reduce *Salmonella* shedding in pigs in an effective, affordable and practical manner.

A second goal of this project was to develop a phage cocktail with increased host range. We again isolated wild-type phages from wastewater treatment facilities and created a library of 10 phages that lysed a

co-culture of *Salmonella* serovars Typhimurium, Kentucky and Enteriditis. These serovars are among the most frequently isolated from food animal products.

The only remaining concern is the fact that our results are at times inconsistent. We are currently characterizing the phages in our library with regards to growth kinetics, killing efficiency and genetic relatedness. We are confident that with more information on the basic properties of these viruses, we can better identify the most appropriate phages for the cocktail and improve the consistency of the product.

Publications Resulting from this Grant:

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