Title: Employing phage therapy to reduce lairage associated increases in *Salmonella* infections and shedding – NPB #06-167

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### II. Industry Summary

Contamination of meat and meat products with foodborne pathogens is usually the result of the carcass coming in contact with the feces of an infected animal during processing. In the case of *Salmonella*, several recent studies have reported that pigs become rapidly infected with the organism during transport and lairage due to *Salmonella* contaminated trailers and holding pens. These infections serve to increase the likelihood of carcass contamination by amplifying the amount of bacteria that enters the processing facility. To counteract these infections, we have developed a phage based anti-*Salmonella* intervention strategy that can be administered to pigs just prior to transport and lairage. We have isolated and characterized 15 wild-type, *Salmonella*-specific phage and developed a means by which the viruses can be microencapsulated for administration to animals. Initial tests of the efficacy of our anti-*Salmonella* phage cocktail were conducted in small pigs (~20-30 pounds) where we treated the pigs with the phage cocktail and then inoculated them with *Salmonella enterica* Typhimurium. Infections in pigs administered the phage treatment were reduced 99.0% to 99.9% in the tonsils, ileum, and cecum. To test our anti-*Salmonella* phage cocktail in a more production-like setting, we infected (in three replicates) four market weight pigs with *Salmonella enterica* Typhimurium. These seeder pigs were allowed to contaminate a holding pen for 48 hours. At 48 hours post-inoculation, 16 naïve pigs (in three replicates) were introduced to the contaminated environment. Eight pigs were given the anti-*Salmonella* phage cocktail while the other eight were mock-treated. Phage treatment reduced cecal and ileal *Salmonella* infections by approximately 95% and 90%, respectively, in phage treated pigs. These data indicate that phage therapy can be used as an effective means to reduce or limit *Salmonella* infections during the crucial periods of transport and lairage. Contact person: Paul Ebner, Asst. Professor, Dept. of Animal Sciences, Purdue University, 915 W. State St., West Lafayette, IN 47907, 765-494-4820, pebner@purdue.edu.
III. Scientific Abstract

Contamination of meat and meat products with foodborne pathogens is usually the result of the carcass coming in contact with the feces of an infected animal during processing. In the case of *Salmonella*, several recent studies have reported that pigs become rapidly infected with the organism during transport and lairage due to *Salmonella* contaminated trailers and holding pens. These infections serve to increase the likelihood of carcass contamination by amplifying the amount of bacteria that enters the processing facility. We conducted a series of experiments to test whether phage therapy could be used to counteract *Salmonella* infections at this crucial period. Fifteen anti-*Salmonella* phage were isolated from various Indiana wastewater treatment facilities and characterized by electron microscopy. In preparation for administration to pigs, the viruses were microencapsulated using a sodium alginate-lysine method. Preliminary experiments done with small pigs (20-30 pounds) indicated that administration of the anti-*Salmonella* phage cocktail at the time of inoculation with *Salmonella enterica* Typhimurium reduced the extent of infection by 99.0-99.9% (2-3 log growth) in the tonsils, ileum, and cecum as compared to mock-treated pigs. To test the efficacy of phage therapy in a more production-like setting, we inoculated four market weight pigs (in three replicates) with *Salmonella enterica* Typhimurium and allowed the infected pigs to contaminate a holding pen for 48 hours. At 48 hours post-inoculation, 16 naïve pigs were introduced to the contaminated environment. Eight pigs were administered the anti-*Salmonella* phage cocktail (orally) while the other eight pigs were mock-treated. All pigs were euthanized at six hours. Treatment with the anti-*Salmonella* phage cocktail significantly reduced cecal infections (95%, $P < .05$) while showing a strong tendency to reduce ileal infection (90%, $P = .06$). Taken together, these data indicate that phage therapy can be used as an effective anti-*Salmonella* intervention strategy to combat holding and lairage-associated increases in *Salmonella* infections.

IV. Introduction

Each year the Centers for Disease Control and Prevention report over 30,000 culture-confirmed cases of *Salmonellosis* in the United States (CDC, 2006). The vast majority of outbreaks are foodborne with contaminated eggs, meat, and dairy products most often implicated (Mead *et al*., 1999). In the case of meat, it is widely accepted that *Salmonella* contamination usually results from the carcass coming in contact with the feces of an infected animal during processing (Morgan *et al*., 1987). As such, there is a need for effective pre-harvest intervention strategies that reduce the amount of *Salmonella* in the animal’s intestine prior to its entry into the abattoir, thereby reducing the risk of the organism coming in contact with meat.

In many food animals these strategies are confounded by the fact that seemingly healthy animals, with no history of *Salmonella* infection, can begin shedding the organism in high concentrations following transport and/or lairage (Williams and Newell, 1970). The increases in pathogen shedding are attributed to stress (Isaacson *et al*., 1999) and/or rapid infections from *Salmonella* contaminated trailers and holding pens (Larsen *et al*., 2004; Hurd *et al*., 2002 Rostagno *et al*., 2003) and are thought to increase the likelihood of *Salmonella* contaminated pork. The problem is exacerbated by the fact that producers are very limited as to the types of antimicrobials that can be administered to pigs in the time period immediately leading up to slaughter.

Here we proposed an intervention strategy aimed at reducing holding pen associated increases in *Salmonella* infections and shedding through the administration of *Salmonella* specific-bacteriophages (phages) just prior to lairage. It was expected that high concentrations of bacteriophages in the animal’s gut at this critical time would counteract any increases in *Salmonella* shedding, thereby decreasing the risk of contamination during processing. Moreover, we aimed to develop a manner in which bacteriophages could be microencapsulated, increasing their efficacy and making their administration easier and more practical.
**Salmonella Infection and Lairage**

Pigs are often described as being reservoirs for *Salmonella*. Indeed, the organisms are ubiquitous in swine environments and can easily be isolated from farms of various sizes with varied management practices. *Salmonella* infections in pigs are often asymptomatic and self-limiting. Once infected, however, an otherwise healthy animal may continue to shed the organism in its feces for weeks and sometimes months (Ebner and Mathew, 2000).

**Transport and lairage associated shedding.** Increases in *Salmonella* shedding prevalence in pigs after transport were first observed over thirty years ago (Williams and Newell, 1970) and, for many years, were thought to result from stress-induced reactivations of old infections. Thus far, however, there is little consistent proof that measurable stress increases *Salmonella* shedding under controlled conditions (Calloway et al., 2006; Stabel and Fedorka-Cray, 2004).

More recent studies comparing *Salmonella* serotypes in different swine environments reported that the *Salmonella* strains found in pigs at the farm often do not match those found in pigs at slaughter, implying that pigs may acquire new infections *en route* to or while waiting in the abattoir (Wondwossen et al., 2004a, 2004b). Moreover, two groups recently demonstrated that common holding areas in processing plants can be highly contaminated with diverse strains of *Salmonella* (Rostagno et al, 2003, Larsen et al., 2003) while a third demonstrated that pigs can become infected with *Salmonella* as little as two hours as measured by the presence of the organism in the distal small intestine (Hurd et al., 2001). As pigs are held in abattoir holding pens for an average of 30min to 10hrs, these studies indicate that increases in infection and shedding rates following transport and lairage may also arise from rapid infections by *Salmonella* found in trailers and/or holding pens.

It should be noted that stress and rapid infection might not be mutually exclusive factors in increasing *Salmonella* shedding. Rather, stress may serve to make the pig more susceptible to infection while contaminated trailers and holding pens provide the *Salmonella* source. As there is very little research examining this possible interaction, it remains only a hypothesis.

**Phage Therapy**

Producers are very limited as to anti-*Salmonella* intervention strategies available for use just prior to transport. The majority of antibacterial chemotherapeutics are not an option due to withdrawal times (Mathew and Ebner, 2004). Likewise, while effective at reducing the incidence of *Salmonella* on the farm, most non-antibiotic/antimicrobial strategies such as pre- and probiotics, are of little proven use once the pig is stressed and in a highly contaminated environment. While diligent disinfection of the holding areas does reduce new infections, several reports suggest that multiple intervention strategies are needed to adequately reduce the incidence of *Salmonella* contamination in processing plants (Alban and Stark, 2005; Beloeil *et al*, 2004; Schmidt *et al*, 2004).

Towards this end, phage therapy may prove very useful. Phages are naturally occurring viruses that infect and destroy bacteria (see Stone, 2002 and Huff *et al.*, 2005 for review). They are classified into two general groups: temperate phages and virulent phages (Fig. 1). Temperate phages infect bacteria and integrate their DNA into the bacterial chromosome becoming prophages. Bacteria carrying temperate phages may replicate for several generations as long as the phages remain in pro-phage

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**Fig. 1. Schematic describing the life cycles of temperate versus virulent bacteriophages.**
states. In contrast, virulent phages generally do not integrate into the bacterial chromosome upon infection. Rather, by using bacterial resources, virulent phages immediately begin to replicate. High levels of replication lead to the lysis, and ultimately the death, of the bacterial cell. The phages are released from the dead bacterial cell and continue a cycle of infecting, lysing, and destroying new bacterial cells.

Phages are, in general, very species-specific and do not infect mammalian cells, making them attractive as antibacterials. Indeed, using phages to treat bacterial infections was first proposed in the 1930’s but the introduction of broad-spectrum antibiotics and their effectiveness in safely treating a wide variety of bacterial infections a decade later made other non-antibiotic therapies seem unnecessary (Stone, 2002). The rise in antibiotic resistance, however, has convinced many research groups to revisit the possibility of using phages against difficult to treat infections such as those caused by Pseudomonas and antibiotic resistant tuberculosis (Danelishvili et al., 2006; Hagens et al., 2004).

**Agricultural use of phage therapy.** As the pressure to curtail the subtherapeutic use of antibiotics in food animals increases, agricultural researchers have also begun to revisit phage therapy and several groups have shown that a properly selected phage can eliminate or limit a bacterial infection if administered at the proper time. Loc Carrillo and co-workers demonstrated that oral administration of phages to Campylobacter infected chickens reduced the concentration of Campylobacter shed in the feces by as much as 5 log_{10} CFU/mL (Loc Carillo et al., 2005). Similar results were reported by Wagenaar et al., also working with Campylobacter infected chickens (Wagenaar et al., 2005) and Fiorentin and co-workers working with Salmonella infected chickens (Fiorentin et al., 2005). Using phage therapy in combination with competitive exclusion has also proved successful in limiting Salmonella infections in chickens (Toro et al., 2005). Unfortunately, there are very few published accounts of using phage therapy in swine production and, to our knowledge, this would be the first attempt at using phages to combat lairage-associated Salmonella infections.

**Benefits of phage therapy.** Phages are thought to be the most abundant life form in nature. As such, perhaps the main benefit of phage therapy is the seemingly endless supply of phages with diverse antibacterial mechanisms of action and the ease with which they can be isolated from common sources such as water, sewage, and soil (McLaughlin et al., 2006; O’Flynn et al., 2006; Ebner unpublished results, 2006 [Fig. 3]). Phages are also easy to prepare as the viruses can be grown to very high titer with little effort and recent reports indicate that they are amenable to further processing such as resuspension in antacid (Loc Carillo et al., 2005). What is more, phages usually only target a certain species of bacteria and in many cases only a specific strain. Therefore, phage therapy does not have the global effect on gut microflora and the subsequent side effects (e.g., diarrhea) commonly seen with antibiotic therapy. As phages are a longtime reagent in the study of genetics, a great deal is known regarding their molecular biology, which allows them to be easily manipulated. Finally, phage therapy may offer a more “green”, and thereby a more consumer-accepted, approach to treating bacterial infections in pigs.

**Limits of phage therapy.** Phage therapy does have limitations and each is deserving of mention. First, the growth promoting effects of including antibiotics in animal feeds are due, at least in part, to the relatively broad spectrum of the drugs. The narrow spectrum of most phages, while previously stated as a benefit, probably makes their use as antibiotic-replacing growth promoters implausible. Moreover, as with antibiotics, bacteria can develop resistance to phages, and in some cases quite rapidly (Loc Carillo et al., 2005). Unlike antibiotics, however, new phages effective at killing resistant bacteria can be isolated in as little as a week (McLaughlin et al., 2006; O’Flynn et al., 2006). Finally, phage therapy is limited by what is referred to as the phage proliferation threshold. Simply stated, phages require bacteria for their own replication. Thus, when populations of the target bacteria drop due to phage mediated-killing, so does the phage concentration. Therefore, after an initial reduction, concentrations
of the target bacteria may increase once again after phage levels drop below a critical threshold (Loc Carillo et al., 2005).

**Phage for preharvest reduction in Salmonella.** Most limits of phage therapy become evident when it is suggested as a long-term, wholesale replacement for traditional antibiotics, for which it is probably ill suited. Phages still have excellent potential for use as antibacterial biotherapies, however, if used within their known limits. Towards this end, combating reactivating or rapid *Salmonella* infections associated with lairage is, perhaps, an ideal use of phage therapy. First, *Salmonella* specific virulent phages are readily isolated from swine effluent lagoons (McLaughlin et al., 2006; O’Flynn et al., 2006; Ebner, unpublished results). Second, the problems associated with resistance development would be negligible as phages would be administered only once and the animals processed shortly thereafter. Third, phages would be delivered in high initial concentrations and only be required to be effective for a very short period of time (~8hrs) thereby making phage proliferation threshold limits and rebounding growth of the target bacteria irrelevant.

V. **Objectives (from original proposal)**

Just prior to processing, pigs often begin shedding *Salmonella* in high concentrations as a result of stress-induced reactivation of pre-existing infections, new infections acquired during transport and lairage, or both. The aim of this project is to determine whether oral administration of *Salmonella* specific phages to pigs prior to their entry into a *Salmonella* contaminated holding pen can effectively reduce *Salmonella* infections and shedding, thereby decreasing the risk of contamination of meat during processing.

VI. **Materials and Methods**

**Phage Isolation and Characterization.** Fifteen *Salmonella enterica* Typhimurium specific lytic phage were isolated from fifteen distinct wastewater treatment facilities using a previously described method (O’Flynn et al., 2006) with minor deviations. Briefly, fecal samples were combined with 50% w/v sterile 2YT medium, added (5mL) to 20mL of early log-phase *Salmonella enterica* Typhimurium γ4232 (*Salmonella* Typhimurium), and incubated for six hours. Phage were separated first by centrifugation and then filter (0.45μm) purified. Putative phage samples (100μL) were then combined with log phase *Salmonella* Typhimurium (100μL), CaCl₂ (100μL), 3mL TSA overlay (0.75% agar), and spread on TSA plates. Plates were incubated overnight and phage were identified by plaque formation. Each phage was characterized by transmission electron microscopy (TEM; Fig. 1).

**Phage Cocktail Microencapsulation.** Phage were combined with sodium alginate, Span-85 (Sigma, St. Louis, MO) and canola oil. The resulting solutions were emulsified and mixed with CaCl₂ and ZnCl₂ creating microspheres. Microspheres were then be separated by centrifugation and resuspended in sterile PBS containing poly-L-lysine (Mittal et al., 2001).

**Preliminary Trials.** One preliminary trial was conducted to gauge the potential efficacy of the phage cocktail. Twelve small pigs (~20-30) were randomly separated into two groups of six. The first group was administered the microencapsulated phage cocktail orally while the second was administered a mock treatment (microencapsulation ingredients without the phage). All pigs were then inoculated with 10⁷ CFU of *Salmonella enterica* Typhimurium (γ4232) which contained a nalidixic acid resistance marker for easier isolation. Each group was then given a second treatment or mock-treatment at three hours post-inoculation. All pigs were euthanized at six hours and tonsil, ileal, cecal, mesenteric lymph node and fecal samples were collected. *Salmonella* was reisolated from each sample using previously described methods (Ebner and Mathew, 2000).
**Main Trials.** Four market weight pigs (in three replicates) were inoculated with $3 \times 10^8$ CFU of *Salmonella enterica* Typhimurium ($\gamma 4232$) and held in a holding pen with feed for 48 hours to allow the infected pigs to create a contaminated environment like those seen in abattoir holding pens. At 48 hours, 16 naïve pigs were introduced to the *Salmonella* contaminated environment. Prior to their introduction the pigs were divided into two groups. The first group received the anti-*Salmonella* phage cocktail orally while the second received the same mock treatment as described above. Infected and naïve pigs were then comingled for six hours. Phage treated pigs received a second and third treatment at two and four hours post co-mingling. All pigs were euthanized at six hours post co-mingling. As described above, ileal, cecal, mesenteric lymph node and fecal samples were collected and *Salmonella* was reisolated from each sample using previously described methods (Ebner and Mathew, 2000). Data were analyzed using Proc Mixed of SAS.

**VII. Results**

**Preliminary Trials.** Twelve 20-30lb. pigs were inoculated with *Salmonella enterica* Typhimurium. Six of the twelve pigs were co-administered the microencapsulated anti-*Salmonella* phage cocktail while the remaining six pigs were co-administered a mock treatment (microencapsulation ingredients without phage). All pigs were euthanized at six hours post-inoculation and tonsil, ileal, cecal, lymph node, and fecal samples were examined for the presence of the challenge *Salmonella* strain. Treatment with the anti-*Salmonella* phage cocktail resulted in significant reductions in the extent of *Salmonella* infection. Tonsil samples from treated pigs contained three logs less *Salmonella* (99.9% reduction) than samples taken from mock-treated pigs (Fig. 2). Likewise, ileal and cecal infections were reduced by two and three logs (99.0% and 99.9%), respectively in phage treated pigs (Fig. 3; Fig 4). Although the sample size for these experiments was very small, we analyzed these data using Proc Mixed of SAS and found significant differences in several of the measurements (Fig. 2; Fig. 3; Fig. 4). Fecal and lymph node samples were not different between the two groups (data not shown).

**Main Trials.** To test the effectiveness of the phage cocktail under more production-like settings, we challenged four market weight pigs with *Salmonella enterica* Typhimurium and allowed the infected pigs to contaminate a holding pen area for 48 hours. At 48 hours, 16 naïve pigs were divided into two groups. Eight pigs were administered the anti-*Salmonella* phage cocktail while the remaining eight pigs received a mock treatment. All pigs were then co-mingled in the *Salmonella* contaminated environment for six hours. At six hours, all pigs were euthanized for sample collection. Administration of the phage cocktail significantly reduced infections in the cecum ($P < .05$; Fig. 5) while showing a strong tendency to reduce infections in the ileum ($P = .06$; Fig 6). Unlike cecal and ileal samples, fecal swab samples and lymph node samples had to be enriched because of low *Salmonella* concentrations. Therefore these samples were not quantified and therefore were not accurate indications of the extent of infections. Nevertheless, fecal and lymph node samples (positive or negative) were not different between the two groups (data not shown).

**VII. Discussion**

These experiments support those done in poultry that showed the promise of using phage therapy as an anti-*Salmonella* therapeutic (Higgins *et al*., 2005; Waganaar *et al*., 2005; Fiorentin *et al*., 2005). To our knowledge, ours is the first attempt to develop phage therapy as a means to counteract transport and lairage associated *Salmonella* infections. Likewise, these are the first experiments to be done on market weight pigs in a more production-like setting. The data indicate that phage therapy can reduce *Salmonella* infections under these conditions and, thus, has great potential to be used as a food safety intervention strategy.
The differences shown in market weight pigs under production-like conditions, while significant, were not as great as those seen in our preliminary trials where smaller pigs were co-administered both the challenge *Salmonella* and the phage cocktail. The trials with market weight pigs were conducted in three replicates in three consecutive weeks. Each replicate was conducted in the same room and while the room was cleaned between replications, complete sterilization was not practical. Examination of the raw data showed that with each replicate, the differences in the level of infection between treated and mock-treated pigs became smaller (Fig. 7). Significant reductions (P < .05) were seen in both the first and second replicate in both ileal and cecal samples. These differences were not seen in the third replicate (Fig. 8; Fig 9). It is entirely possible, if not probable, that with time the challenge *Salmonella* strain became resistant to the phage cocktail. Thus, the pigs were exposed to greater concentrations of phage resistant *Salmonella* from the room itself with each replicate. We are currently examining the resistance levels in recovered *Salmonella* samples to test this hypothesis.

Regardless, these data show that phage therapy can be used effectively to limit transport and lairage-associated increases in *Salmonella* infections in pigs. In these experiments, treated pigs were administered the phage cocktail by *gavage*. Future experiments will focus on administering the phage through water systems or in the feed making the treatment more practical. As we are able to effectively microencapsulate the phage, both water or feed-based systems are very feasible. Likewise, the problem of resistance development could be easily circumvented by rotating the composition of the phage cocktail, i.e., interchanging the actual strains used to make the treatment. This would, of course, require a much larger phage library, but as phage are the most abundant organisms on earth and are easily isolated from several different common sources, building such a library is entirely possible.

VII. Works Cited


Fiorentin L, Vieira ND, Baronia W. 2005: Oral treatment with bacteriophages reduces the concentration of Salmonella enteriditis PT4 in caecal contents of broilers. Avian Path. 34:258-263.


Fig. 1. Isolation and Identification of anti-Salmonella Phage. Fifteen anti-Salmonella phage were individually isolated from fifteen different wastewater treatment plants throughout Indiana. Image is of one phage sample taken by transmission electron microscopy (TEM).

Fig. 2. Phage therapy reduces Salmonella infections in tonsils of treated pigs. In preliminary trials, small pigs (20-30lbs.; n=6 per treatment) were coadministered Salmonella enterica Typhimurium and an anti-Salmonella cocktail. Tonsil samples were collected by scraping and Salmonella was enumerated. Phage treatment reduced infections in the tonsils by approximately 99.9%. Bars with different subscripts are statistically different at P < .05.
Fig. 3. Phage therapy reduces *Salmonella* infections in ileum of treated pigs. In preliminary trials, small pigs (20-30lbs.; n=6 per treatment) were coadministered *Salmonella enterica* Typhimurium and an anti-*Salmonella* cocktail. Ileal samples were collected and *Salmonella* was enumerated. Phage treatment reduced infections in the tonsils by approximately 99.0%. Bars with different subscripts are statistically different at $P < .05$.

Fig. 4. Phage therapy reduces *Salmonella* infections in the cecum of treated pigs. In preliminary trials, small pigs (20-30lbs.; n=6 per treatment) were coadministered *Salmonella enterica* Typhimurium and an anti-*Salmonella* cocktail. Cecal samples were collected and *Salmonella* was enumerated. Phage treatment reduced infections in the tonsils by approximately 99.9%; bars with different subscripts are statistically different at $P < .05$. 
Fig. 5. Phage therapy reduces *Salmonella* infections of the cecum under production-like settings. Four market weight pigs (in three replicates) were inoculated with *Salmonella enterica* Typhimurium and held in a common pen. At 48 hours post-inoculation, 16 naïve pigs were divided into two groups receiving either an anti-*Salmonella* phage cocktail or a mock-treatment. All pigs were co-mingled in the *Salmonella* contaminated pen for 6-8 hours. Cecal samples were collected and *Salmonella* was enumerated. Treatment with the phage cocktail significantly reduced *Salmonella* infections in treated pigs (P < 0.05).
Fig. 6. Phage therapy reduces *Salmonella* infections of the ileum under production-like settings. Four market weight pigs (in three replicates) were inoculated with *Salmonella enterica* Typhimurium and held in a common pen. At 48 hours post-inoculation, 16 naïve pigs were divided into two groups receiving either an anti-*Salmonella* phage cocktail or a mock-treatment. All pigs were co-mingled in the *Salmonella* contaminated pen for 6-8 hours. Ileal samples were collected and *Salmonella* was enumerated. Treatment with the phage cocktail showed a strong tendency to reduce *Salmonella* infections in treated pigs (P = 0.06).

Fig. 7. *Salmonella* may acquire resistance to the phage cocktail with time. Data from cecal samples are separated by replication to illustrate the decreasing efficacy of the treatment with time due possibly to increasing resistance to the phage cocktail as described in the discussion section; Phage 1 = phage treatment, first replication.
Fig. 8. *Salmonella* may acquire resistance to the phage cocktail with time. Data from cecal samples are separated by replication and comparisons made within replication. Bars with different subscripts are statistically different at P < .05; Phage:1 = phage treatment, first replication.

Fig. 9. *Salmonella* may acquire resistance to the phage cocktail with time. Data from ileal samples are separated by replication and comparisons made within replication. Bars with different subscripts are statistically different at P < .05; Phage:1 = phage treatment, first replication.