

## Isolation of *Salmonella* spp. and bacteriophage active against *Salmonella* spp. from commercial swine.

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### Abstract

Bacteriophage are viruses that prey on bacteria and may be a potential strategy to reduce foodborne pathogenic bacteria in the gastrointestinal tract of food animals. Phages are fairly common in the gastrointestinal microbial ecosystem of mammals, but the incidence is unknown. If phage are to be an intervention strategy, we must understand their role in the microbial ecology of the gut. From a regulatory perspective, knowing incidence of phage is crucial. Therefore the current study was designed to determine the incidence of phage active against *Salmonella* spp. in the feces of commercial finishing swine in the United States. Fecal samples (n=60) were collected from each of six commercial swine finishing operations. Samples were collected from 10 randomly selected pens throughout each operation. Total number of fecal samples collected in this study was n=360. *Salmonella* spp. were found in 6.6% of the fecal samples. *Salmonella* spp. were isolated from only 2 farms and the serotypes represented were Schwarzengrund, Anatum, Ohio and Heidelberg. Bacteriophages were isolated from fecal sample through 2 parallel methods, 1) initial enrichment in *Salmonella* Typhimurium, or 2) initial enrichment in *E. coli* B (a strain very sensitive to phages); followed by direct spot-testing against *Salmonella* Typhimurium. Bacteriophages active against *Salmonella* Typhimurium were isolated from 1.1% (4/360) of the individual fecal samples when initially enriched in *Salmonella* Typhimurium, but *E. coli* B-killing phages were isolated from 43.8% (158/360) of the fecal samples but only 2 of these isolates were capable of killing *Salmonella* Typhimurium. Our results indicate that bacteriophage capable of killing *Salmonella* Typhimurium are fairly widespread across commercial swine production facilities but may be present at relatively low populations. These results indicate that phage (predator) populations may vary along with *Salmonella* (prey) populations and that phage could potentially be used as a food safety pathogen reduction strategy.

### Introduction

Food-borne *Salmonella* infections in the United States are estimated to cost the economy \$2.4 billion annually (ERS/USDA, 2001). Approximately 6-9% of human salmonellosis is associated with the consumption of pork products (Frenzen et al., 1999). *Salmonella* is relatively common on swine farms and has been isolated from all stages of the pork production chain (Davies et al., 1999; Fedorka-Cray et al., 1997; Rostagno et al., 2003). *Salmonella* is a threat to the pork industry not only from a food-safety perspective as a public health concern, but some *Salmonella* serotypes can cause clinical illnesses in swine, negatively impacting production efficiency and profitability (Schwartz, 1991).

Bacteriophage are viruses that specifically infect bacteria and reproduce within them, killing the host bacterium through cellular lysis caused by the release of daughter phages. Phage were widely used in eastern Europe in place of antibiotics and have been called an "infectious cure for infectious disease" (Barrow, 2001). Due to increasing concerns about antibiotic resistance linkage to animal agriculture, considerable research has been focused on finding alternatives to antibiotics to reduce pathogens in food animals. Because phage exhibit a high degree of specificity for target bacteria it has been suggested that bacteriophage be used as a "designer antimicrobial" to eliminate specific pathogens from the gastrointestinal microbial population, including *Salmonella*,

*Campylobacter*, *Listeria* and *E. coli* O157:H7 (Alisky et al., 1998; Loc Carrillo et al., 2005; Smith and Huggins, 1983).

Bacteriophage have been long known to be members of the intestinal microbial consortium. But the role they play in the ecology of the gut has been unclear; theories have abounded about preventing overgrowth to a role in diurnal variation to a role in nutrient cycling. However the exact incidence of these bacterial predators has never been fully investigated. Most research involving phages in the intestinal tract of animals has been exclusively qualitative in nature; incidence rates were determined using less than 20 animals of various species (Dhillon et al., 1976). Therefore this study was conducted to determine the incidence of phage in swine, by examining two separate issues, 1) what is the incidence of phage that kills *Salmonella* Typhimurium (one of the most important human illness serotypes in the United States), and 2) what is the overall incidence of bacteriophage in commercial swine.

## Materials and Methods

Fresh fecal samples (approximately 100 g from a single source; n = 6 samples per pen) were collected from each of 10 finishing pens per commercial swine farm (n = 10 pens/farm; n = 60 fecal samples/farm). Total number of fecal samples collected in this study was n=360. All samples were collected within a 45 min period immediately after the morning feeding. Immediately upon collection, samples were individually bagged in sealed whirl-pak bags after collection and kept on ice during transport prior to analysis (for approximately 24 h).

To qualitatively enrich for *Salmonella* populations, 3 g of feces were added to tubes containing 27 mL of tetrathionate broth (Difco Laboratories) and incubated at 37 °C for 24 h. After this incubation, 200 µL of the tetrathionate enrichment were added to 5 mL Rapport-Vassilidis R10 broth and incubated an additional 24 h at 42 °C before being streak-plated onto brilliant green agar (BGA) supplemented with novobiocin (25 µg/mL). The BGA<sub>Nov</sub> plates were incubated for 24 h at 37 °C; colonies that exhibited typical *Salmonella* morphology were individually picked for further physiological characterization and were inoculated onto Triple Sugar Iron (TSI) agar slants and Lysine Iron agar (LIA) slants (Difco, Inc.). Each slant was incubated at 35 °C for 24 h. *Salmonella*-positive samples were confirmed by slide agglutination using SM-O antiserum poly A-I and V-I, and group C1 factors. *Salmonella* isolates were stored in glycerol and TSB at -80 °C until confirmatory serotyping was performed by the National Veterinary Services Laboratory (NVSL) in Ames, IA.

**Bacteriophage enrichment and isolation.** Fecal samples were screened for the presence of *Salmonella* Typhimurium bacteriophage. Feces (1 g) were mixed in sterile conical tubes containing 9 ml of phosphate buffered saline (pH 6.8). Chloroform (0.5 ml) was added to each tube and tubes were thoroughly mixed before being allowed to stand at 24 °C for 2 h. The top layer from this tube was removed and placed in a new sterile tube containing 0.5 ml chloroform. Portions (0.3 ml) of the chloroform-free top layer were mixed with 1.2 ml volumes of early-log-phase (< 0.2 OD) *S. Typhimurium* or *E. coli* B (10<sup>8</sup> CFU/ml, grown at 39 °C) and were incubated in anoxic TSB broth in sealed Hungate tubes overnight at 39 °C. *E. coli* B was used in this study as an initial propagation strain because, 1) it is susceptible to bacteriophage of several types, and 2) use of this strain to propagate natural bacteriophage allows us to detect a broader range of phage in an initial bacteriophage activity screening. Samples (1.5 ml) were collected and added to tubes containing 0.2 ml of chloroform for 30 min. These samples were subsequently centrifuged at 19,000 x g in a microcentrifuge for 10 min. The top layer of the supernatant was removed, and stored in a fresh sterile tube following sterilization by filtration through a 0.2 µm filter. Samples were subjected to a plaque assay (Sambrook and Russell, 2001) using *S. Typhimurium* or *E. coli* strain B as the propagation host and grown on TSB plates incubated anaerobically. Plates were incubated overnight at 39 °C.

**Spectrum of bacteriophage activity.** All bacteriophage plaques purified from the *S. Typhimurium* and *E. coli* B plates (3 plaques/sample) were assessed for their ability to form plaques on a range of intestinal bacteria. *E. coli* F18 and K88 were obtained from the FFSRU culture collection. Other bacterial species tested for bacteriophage activity included *Salmonella derby*, *S. typhimurium*, *S. dublin*, *S. enteritidis*, *S. choleraesuis*, *S. montevideo*, *S. mbandaka*, *Enterococcus faecalis*, *Enterococcus faecium* and *E. coli* O157:H7 from the FFSRU culture collection. Each bacterial strain was grown on TSB plates incubated anaerobically and were exposed to an equal amount of bacteriophage plaque forming units (PFU) of each bacteriophage isolate. The

bacteriophage that were isolated in this study are currently being genetically and physiologically characterized and further characteristics of the bacteriophage will be reported in future studies.

**Data Analysis.** Point prevalence of *Salmonella* and bacteriophage shedding was calculated individually by dividing the number of pathogen culture-positive fecal samples by the total of samples collected per farm ( $n = 60$  per farm, 360 total samples). Correlations of prevalence were using Epi Info 6.0, but due to the relatively low numbers of pens and incidences in this study, no correlations were found. Length of time spent in pen or farm were not included in the models because the record-keeping was not complete or available to the researchers.

## Results

*Salmonella* Enterica serotypes were found in 6.6% of the fecal samples (24/360). *Salmonella* spp. were isolated from only 3 of the 6 farms and the serotypes represented were Schwarzengrund, Anatum, Derby, Ohio and Heidelberg (Table 1).

**Table 1.** *Salmonella enterica* serotype, serogroup, phage active against *Salmonella* Typhimurium, and phage active against *E. coli* strain B isolated from commercial finishing swine in the central United States.

Farm	Serotype (number)	Serogroup	Phage + on <i>S.</i> Typh	Phage + on <i>E. coli</i> B
A	Anatum (1) Derby (1)	E1 B	0	0
B	None		3	18
C	None		0	13
D	None		1	7
E	Ohio (3) Heidelberg (1)	C1 B	2 (after B enriched)	60
F	Schwarzengrund (14) Anatum (4)	B E1	0	60
<b>Total</b>	<b>24/360</b>		<b>6/360</b>	<b>158/360</b>

Bacteriophages were isolated from each fecal sample through 2 parallel methods, 1) initial enrichment in *Salmonella* Typhimurium, or 2) initial enrichment in *E. coli* B (a strain very sensitive to phages) followed by direct spot-testing against *Salmonella* Typhimurium. Bacteriophages active against *Salmonella* Typhimurium were isolated from 1.6% (6/360) of the total individual fecal samples, but *E. coli* B-killing phages were isolated from 43.8% (158/360) of the fecal samples. Only 2 of the *Salmonella*-killing phage were isolated from samples that were first enriched in *E. coli* B. All of these phages created clearing zones when plated onto *S. Typhimurium* and were characterized by their pattern against other *Salmonella* serotypes (data not shown). However, the spectrum of *Salmonella*-killing activity was very narrow, with only one of the phage killing another *Salmonella* serotype than Typhimurium (Derby).

## Discussion

*Salmonella* spp. and other foodborne pathogenic bacteria can live in the gut of mammals, including swine. A wide variety of *Salmonella* serotypes have been isolated from swine around the world. The present study indicates that *Salmonella* are present in commercial finishing operations in the U.S. at a relatively low incidence that is comparable to other published surveys (Davies et al., 1999; Morrow et al., 1999). However, the fact that *Salmonella* are isolated from apparently healthy finishing swine has serious implications for pork safety; yet of the serotypes isolated in this study, only Anatum is found in the most common human isolates of the CDC. It is important to note that less than 7% of the fecal samples were positive for *Salmonella* spp., and these were limited to 3 of the 6 farms surveyed, indicating that herd health measures have indeed been effective in reducing the incidence of *Salmonella* in finishing swine.

Phages are normal members of the microbial ecosystem of the gastrointestinal tract of animals and humans, and are commonly isolated from community wastewater streams. In spite of understanding that phage are widespread in nature, no research has been performed to estimate the incidence of phage in food animals until recently, and never before in commercial swine. The

widespread nature of phage that were active against *E. coli* B was surprising, but the incidence varied between farms, from being ubiquitous on 2 farms to completely absent on one farm.

Phage that killed *Salmonella* Typhimurium were not as widespread on farms; only 6 out of the 360 samples tested positive for phage active against *S. Typhimurium*. Phage active against *S. Typhimurium* had a very narrow activity spectrum, and did not affect a variety of other *Salmonella* serotypes. Phage specific to *S. Typhimurium* were not widespread on these farms, likely because *S. Typhimurium* is not widespread on the farms for a *S. Typhimurium* phage to prey upon. These data suggest that in order to utilize phage to reduce *Salmonella* in swine, that a specific phage or phages be isolated for each specific serotype or group of related serotypes.

Phage have been suggested as a mechanism to reduce *Salmonella* spp. contamination in swine as an animal health adjunct, or as a potential preharvest intervention strategy. It appears that this strategy may be more difficult than previously considered due to the relatively narrow spectra of phage activity against *Salmonella* serotypes. In order to reduce *Salmonella* in the U.S. swine population, *Salmonella*-killing phage that affect the serotypes of interest must be isolated from several swine sources to reduce the possibility of resistance development and to ensure that the phage are effective against all strains of the serotypes of interest.

## Conclusions

Our results indicate that bacteriophage are fairly widespread across commercial swine production facilities, but they may be present at relatively low populations. Phage capable of killing *Salmonella* Typhimurium are found in commercial swine, but were not found at a high incidence. This is potentially due to a predator/prey cycle between the phage (predator) and *Salmonella* (prey) populations. These results suggest that because this cycle naturally exists in the commercial environment, that phage could potentially be used as a food safety pathogen reduction strategy. However, further research is needed to understand the spectrum of activity of each phage type, and to specifically isolate phages active against the *Salmonella* spp. that most directly affect swine production efficiency, animal morbidity/mortality, and food-borne illness.

## References

- ALISKY, J., ICZKOWSKI, K., TROITSKY, N. 1998. Bacteriophages show promise as antibacterial agents. *Journal of Infection* 36, 5-15.
- BARROW, P. 2001. The use of bacteriophages for treatment and prevention of bacterial disease in animals and animal models of human infection. *Journal of Chemistry Technology and Biotechnology* 76, 677-682.
- DAVIES, P., FUNK, J., MORROW, M. 1999. Fecal shedding of *Salmonella* by a cohort of finishing pigs in north carolina. *Swine Health and Production* 7, 231-234.
- DHILLON, T., DHILLON, E., CHAU, H., LI, W., TSANG, A. 1976. Studies on bacteriophage distribution: Virulent and temperate bacteriophage content of mammalian feces. *Applied and Environmental Microbiology* 32, 68-74.
- ERS/USDA. 2001. ERS estimates foodborne disease costs at \$6.9 billion per year No. 2004. Economic Research Service/United States Department of Agriculture. <http://www.ers.usda.gov/Emphases/SafeFood/features.htm>
- FEDORKA-CRAY, P., HOGG, A., GRAY, J., LORENZEN, K., VELASQUEZ, J., VON BEHREN, P. 1997. Feed and feed trucks as sources of *Salmonella* contamination in swine. *Journal of Swine Health Production* 5, 189-193.
- FRENZEN, P., BUZBY, J., ROBERTS, T. 1999. An updated estimate of the economic costs of human illness due to foodborne *Salmonella* in the United States. In, 3rd Int. Symp. on the Epidemiology and Control of *Salmonella* in Pork, Washington, DC. p 215-218.
- LOC CARILLO, C., ATTERBURY, R., EL-SHIBINY, A., CONNERTON, P., DILLON, E., SCOTT, A., CONNERTON, I. 2005. Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. *Applied and Environmental Microbiology* 71, 6554-6563.
- MORROW, W., DAVIES, P., SEE, T., EISEMANN, J., ZERING, K., KIHSTROM, S., KARLI, K. 1999. Prevalence of *Salmonella* spp. In the feces on farm and ceca at slaughter for a cohort of finishing pigs. In, 3rd Int. Symp. Epidemiol. Control of *Salmonella* in Pork, Washington, D. C. p 155-157.

ROSTAGNO, M., HURD, H., MCKEAN, J., ZIEMER, C., GAILEY, J., LEITE, R. 2003. Preslaughter holding environment in pork plants is highly contaminated with *Salmonella enterica*. Applied and Environmental Microbiology 69, 4489-4494.

SCHWARTZ, K. 1991. Salmonellosis in swine. In: The compendium on continuing education for the practicing veterinarian No. 13. p 139-147.

SMITH, H., HUGGINS, R. 1983. Effectiveness of phages in treating experimental *Escherichia coli* diarrhoea in calves, piglets and lambs. Journal of General Microbiology 129, 2659-2675.