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Occurrence of *Salmonella*-Specific Bacteriophages in Swine Feces Collected from Commercial Farms

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Abstract

Salmonella is one of the leading causes of human foodborne illness and is associated with swine production. Bacteriophages are naturally occurring viruses that prey on bacteria and have been suggested as a potential intervention strategy to reduce *Salmonella* levels in food animals on the farm and in the lairage period. If phages are to be used to improve food safety, then we must understand the incidence and natural ecology of both phages and their hosts in the intestinal environment. This study investigates the incidence of phages that are active against *Salmonella* spp. in the feces of commercial finishing swine. Fecal samples ($n = 60$) were collected from each of 10 commercial swine finishing operations. Samples were collected from 10 randomly selected pens throughout each operation; a total of 600 fecal samples were collected. *Salmonella* spp. were found in 7.3% (44/600) of the fecal samples. Bacteriophages were isolated from fecal samples through two parallel methods: (1) initial enrichment in *Salmonella* Typhimurium; (2) initial enrichment in *Escherichia coli* B (an indicator strain), followed by direct spot testing against *Salmonella* Typhimurium. Bacteriophages active against *Salmonella* Typhimurium were isolated from 1% (6/600) of the individual fecal samples when initially enriched in *Salmonella* Typhimurium, but *E. coli* B-killing phages were isolated from 48.3% (290/600) of the fecal samples and only two of these phages infected *Salmonella* Typhimurium on secondary plating. Collectively, our results indicate that bacteriophages are widespread in commercial swine, but those capable of killing *Salmonella* Typhimurium may be present at relatively low population levels. These results indicate that phages (predator) populations may vary along with *Salmonella* (prey) populations; and that phages could potentially be used as a food safety pathogen reduction strategy in swine.

Introduction

Foodborne *Salmonella* infections have been estimated to cost the U.S. economy $2.4 billion annually (USDA-ERS, 2001). More than 1.3 million illnesses and over 500 deaths are attributed to this pathogenic organism yearly (Mead *et al.*, 1999; USDA-ERS, 2001), and approximately 6%–9% of the U.S. human illnesses are associated with 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 process (Fedorka-Cray *et al.*, 1997; Davies *et al.*, 1999; Rostagno *et al.*, 2003). *Salmonella* is a threat to the pork industry not only from the perspective of food safety, but also as a broader public health concern. Further, some of the most common swine-associated *Salmonella* serotypes can also cause clinical illnesses in swine, negatively impacting production efficiency and profitability (Schwartz, 1991).

Owing to increasing concerns regarding the perceived link between antibiotic resistance in human pathogens and antibiotic use in animal agriculture (Sunde *et al.*, 1998; Wondwossen *et al.*, 2000; Salyers and Shoemaker, 2006), considerable research has been focused on finding alternatives to the use of antibiotics to reduce *Salmonella* and other pathogens in swine (Callaway *et al.*, 2007). Bacteriophages are naturally occurring viruses that specifically infect bacteria and reproduce within them, killing the host bacterium through cellular lysis caused by the release of daughter phages

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(Barrow and Soothill, 1997; Summers, 2001). Phages have been widely used in human medicine in Eastern Europe instead of antibiotics for over 80 years and have been called the "infectious cure for infectious disease" (Barrow, 2001). The high degree of selectivity phages exhibit for their bacterial hosts offers the potential for a targeted treatment in which specific pathogens (such as *Salmonella* and *Campylobacter*) are removed from the gastrointestinal microflora (Higgins *et al.*, 2005; Loc Carrillo *et al.*, 2005; Jamalludeen *et al.*, 2009).

Bacteriophages have long been known to be key members of the intestinal microbial consortium (Adams *et al.*, 1966; Dhillon *et al.*, 1976), including that of swine. The role that phages play in the gastrointestinal tract microecology is unclear, as is the incidence of these bacterial predators in the real world. 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; Kai *et al.*, 1985; Klieve and Bauchop, 1988). Therefore, this study was conducted to determine the incidence of naturally occurring bacteriophages in commercial swine by examining two separate issues: (1) What is the incidence of bacteriophages in commercial swine? (2) What is the incidence of phages that kills the foodborne pathogen *Salmonella* Typhimurium?

Materials and Methods

Sample collection

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) from a total of 10 farms for 3 months. Total number of fecal samples collected in this study was 600 samples representing 600 different swine. All samples were collected within the same 45-min period each morning. Immediately upon collection, samples were individually bagged in sealed whirl-pak bags and kept on ice for 24 h during transport to our laboratory.

Qualitative Salmonella *enrichment and identification*

To qualitatively enrich for *Salmonella* populations, 3 g of feces from each sample was added to tubes containing 27 mL of tetrathionate broth (Difco Laboratories, Sparks, MD) and incubated at 37°C for 24 h. After this incubation, 200 μL of the tetrathionate enrichment was added to 5 mL Rapport-Vassilidis R10 broth and incubated for an additional 24 h at 42°C before being individually streak-plated onto brilliant green agar (BGA; Oxoid, Basingstoke, United Kingdom) supplemented with novobiocin (25 μg/mL). The BGA_{Nov} 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 agar slants and lysine iron agar slants (Difco). 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. Putative *Salmonella* isolates were stored in glycerol and tryptic soy broth (TSB) at -80°C until confirmatory serotyping was performed by the U.S. Department of Agriculture (USDA)–National Veterinary Services Laboratory in Ames, IA.

Bacteriophage enrichment and isolation

Fecal samples were screened for the presence of *Salmonella* Typhimurium bacteriophages using two parallel screening enrichments. 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 in parallel with 1.2 mL volumes of early-log-phase (<0.2 optical density) *Salmonella* Typhimurium or *Escherichia coli* B (each bacteria at 10^8 CFU/mL, grown at 39°C) and were incubated in anoxic TSB broth in sealed anoxic Hungate tubes overnight at 39°C. *E. coli* B was used as a parallel enrichment method as a general indicator for phages because of its broad sensitivity to several types of phages, and the use of a very sensitive strain can detect lower bacteriophage concentrations in the environment. 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 g in a microcentrifuge for 10 min. The top layer of the supernatant was removed, and stored in a fresh sterile tube after sterilization by filtration through a 0.2 μm filter. Fluid samples containing phage were subjected to a spot test assay (Sambrook and Russell, 2001) by spotting 10 μL onto bacterial lawns of *Salmonella* Typhimurium or *E. coli* strain B and incubated anaerobically overnight at 39°C.

Spectrum of bacteriophage activity

All bacteriophage plaques purified from the *Salmonella* Typhimurium and *E. coli* B plates (three plaques/sample) were assessed for their ability to form plaques on a range of intestinal bacteria. *E. coli* F18, K88, and K12 were obtained from the Food and Feed Safety Research Unit (FFSRU) culture collection. Other bacteria used in screening assays for bacteriophage activity included *Salmonella* Derby, *Salmonella* Typhimurium, *Salmonella* Dublin, *Salmonella* Enteriditis, *Salmonella* Cholerasuis, *Salmonella* Montevideo, *Salmonella* 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 was exposed to an approximately equal amount of plaque forming units of each bacteriophage isolate.

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, 600 total samples). Correlations of prevalence were calculated using Epi Info 6.0 (Center for Disease Control, Decatur, GA), but due to the relatively low numbers of pens and incidences in this study, no correlations were found. Time spent in pen or farm was not included in the models because the record-keeping was not complete or available.

Results

Salmonella enterica serotypes were found in 7.3% of the fecal samples (44/600). *Salmonella* spp. were isolated from 6 of the

Table 1. *Salmonella enterica* Serotype and Bacteriophages Active Against *Salmonella* Typhimurium or *Escherichia coli* Strain B Isolated from Commercial Finishing Swine in the Central United States

Farm	*Serotype (number; percentage)*	*Serogroup*	*Phage + on* Salmonella *Typhimurium*	*Phage + on* E. coli *B (number; percentage)*
A	Anatum (1; 2%) Derby (1; 2%)	E1 B	0	0
B	None		3	18; 30%
C	None		0	13; 21.6%
D	None		1	7; 11.7%
E	Ohio (3; 5%) Heidelberg (1; 2%)	C1 B	0	60 (2 were active against *Salmonella* Typhimurium after initial enrichment); 100%
F	Schwarzengrund (14; 23.3%) Anatum (4; 6.7%)	B E1	0	60; 100%
G	Copenhagen (1; 0.2%)	B	2	60; 100%
H	Johannesburg (13; 21.7%)	B	0	14; 23.3%
I	Typhimurium (2; 3%) Copenhagen (4; 5%)	B B	0	43; 71.6%
J	None		0	15; 25%
Total	44/600 (7.3%)		6 (8[a])/600	290/600 (48.3%)

[a]Represents total after scanning of all phage-positive samples grown on *E. coli* B.

10 farms surveyed, and the serotypes represented were Anatum, Derby, Copenhagen, Heidelberg, Johannesburg, Ohio, Schwarzengrund, and Typhimurium (Table 1). Bacteriophages were isolated from each fecal sample using two parallel methods: (1) initial enrichment in *Salmonella* Typhimurium; (2) initial enrichment in *E. coli* B (a strain very sensitive to phages), which was followed by direct spot testing against *Salmonella* Typhimurium. Bacteriophages active initially against *Salmonella* Typhimurium were isolated from 1.3% (6/600) of the total individual fecal samples that were from three farms (Table 1). Phages that could lyse *E. coli B* were isolated from 48.3% (290/600) of the fecal samples from 90% of the farms. However, only two of the indicator strain *E. coli* B–isolated phages were capable of killing *Salmonella*.

All of the *Salmonella*-killing phages created clearing zones (<5 mm) when plated onto *Salmonella* Typhimurium and were characterized by a narrow spectrum of *Salmonella*-killing activity, with only one of the phages killing another *Salmonella* serotype than Typhimurium (Derby and Mbandaka). Of all other bacterial species tested, only *E. coli* F18 was killed by more than 3% of the isolates; more than 19% of phages isolated from *E. coli* B lysed *E. coli* F18 (Table 2).

Table 2. Bacterial Species Lysed by Phage Isolates from Enrichments Performed in Indicator *Escherichia coli* B Strain ($n = 290$)

Species examined	*Phage isolates active against other species*
Salmonella Cholerasuis	0
Salmonella Derby	1
Salmonella Dublin	0
Salmonella Enteritidis	0
Salmonella Montevideo	0
Salmonella Mbandaka	1
Salmonella Typhimurium	2
Enterococcus faecalis	0
Enterococcus faecium	1
E. coli O157:H7	0
E. coli F18	46
E. coli K88	1
E. coli K12	2

Discussion

Salmonella spp. are some of the most common agents of foodborne human infection in the United States (Mead *et al.*, 1999). *Salmonella* can live in the gastrointestinal tract of swine (Davies *et al.*, 1997; Funk *et al.*, 2001), and can be freely passed via nose-to-nose contact between pigs (Proux *et al.*, 2001), as well as through environmental contamination on farm and in lairage (Fedorka-Cray *et al.*, 1997; Rostagno *et al.*, 2003; Rodriguez *et al.*, 2006). This distribution and transmissibility allows *Salmonella* to be widely disseminated on a farm or when swine enter a slaughter plant. A wide variety of *Salmonella* serotypes have been isolated from swine around the world (Letellier *et al.*, 1999), some of which cause illness in pigs (Schwartz, 1991), but other serotypes can be transmitted to human consumers and cause foodborne illness (CDC, 2006). Recent studies, however, have found that virulence between *Salmonella* Typhimurium isolates from humans and those of animal origin is often distinct (Heithoff *et al.*, 2008).

The present study indicates that *Salmonella* are present in commercial finishing operations in the United States at a relatively low incidence, comparable to other published surveys (up to 19% incidence) (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. Of the serotypes isolated in this study, only Anatum and Typhimurium are found in the most common human isolates of the CDC (2006). It is important to note in the present study that less than 8% of the fecal samples and 50% of the farms were positive for *Salmonella* spp.

Phages are normal members of the microbial ecosystem of the gastrointestinal tract of animals and humans and are commonly isolated from community wastewater streams and animal feces (Tanji *et al.*, 2003; Dumke *et al.*, 2006; Oot *et al.*, 2007). In spite of the acceptance that phages are widespread in nature, little research has been performed to estimate the incidence of phages in food animals until recently (Dhillon *et al.*, 1976; Atterbury *et al.*, 2003), and never before in commercial swine feces. Phages have been isolated from various types of swine manure lagoons, indicating their ubiquity in the swine environment (McLaughlin *et al.*, 2006; McLaughlin and King, 2008). Because of their predatory nature, phages have been suggested as a mechanism to reduce *Salmonella* spp. contamination in food animals, as an animal health adjunct and as a potential preharvest intervention strategy (Greer, 2005; McLaughlin, 2006; Johnson *et al.*, 2008).

Bacteriophages have been used primarily in animals to control diseases such as *E. coli* diarrhea in calves, pigs, and lambs (Smith and Huggins, 1983, 1987; Barrow *et al.*, 1998). Phage treatment decreased enteropathogenic *E. coli* by 5 $\log_{10}$, and reduced mortality in treated calves compared with non-phage-treated controls (Smith and Huggins, 1983, 1987). Bacteriophage therapy has reduced *E. coli* diarrhea in rabbits (Reynaud *et al.*, 1992), *E. coli* septicemia and meningitis in chickens and calves (Barrow *et al.*, 1998), and *E. coli* air saculitis in broiler chickens (Huff *et al.*, 2002). Foodborne pathogenic bacteria such as *Salmonella* and *Campylobacter* have been successfully reduced in experimental studies (Connerton *et al.*, 2004; Higgins *et al.*, 2005; Loc Carrillo *et al.*, 2005). The U.S. Food and Drug Administration has approved the use of phage treatment as an anti-Listerial on meat and poultry products, and has approved the use of phages as a hide-spray against *E. coli* O157:H7 on cattle (FDA, 2006; Omnilytics, 2007). To date, there has been limited use of phage therapy to reduce foodborne pathogenic bacteria in live animals.

The widespread nature of phages active against *E. coli* B in our swine feces was surprising, but the incidence varied widely between farms—ranging from being ubiquitous on two farms to completely absent on one farm. Our research group has previously examined the incidence of phages from feces of range sheep and feedlot cattle. Sheep transported from open rangeland were found to naturally harbor *E. coli* O157:H7–killing bacteriophages (Callaway *et al.*, 2003, 2006; Raya *et al.*, 2006). In feedlot cattle, 15% of the individual fecal samples were positive for *E. coli* O157:H7–infecting bacteriophages and over half of the pens (55%) were positive (Callaway *et al.*, 2006). This result compares favorably with the present study in which generic lytic phages were present in 48% of swine fecal samples.

Bacteriophages specifically recognize their host bacteria, and can target below the species level, sometimes infecting only a few strains within a species. This degree of specificity has led to bacteriophages being used to treat many kinds of human infections, especially in Eastern Europe. In our study, phages that killed *Salmonella* Typhimurium were not as widespread on farms; only 8 out of the 600 samples tested positive for phage active against *Salmonella* Typhimurium, likely because *Salmonella* Typhimurium is not widespread on the present farms for a *Salmonella* Typhimurium phage to prey upon. Phages active against *Salmonella* Typhimurium had a very narrow activity spectrum and did not affect a variety of other *Salmonella* serotypes, including other group B serotypes. These data suggest that to utilize phages to reduce *Salmonella* in swine, a specific phage (or phages) be isolated for each specific serotype or group of related serotypes that are targeted.

Bacteriophages and their targeted bacteria exist in a predator–prey relationship. Therefore, it is crucial to understand what role bacteriophages play in the natural microbial ecology of the animal before we use bacteriophages to eliminate foodborne pathogens in food animals prior to slaughter. Although no statistical relationship was found between the presence of *Salmonella* and *Salmonella* Typhimurium–infecting bacteriophages in feces in this study, it should be noted that only four pigs shed both *Salmonella* and *Salmonella* Typhimurium–infecting bacteriophages simultaneously, and none of these phages were on the farm positive for *Salmonella* Typhimurium (Table 1). This could indicate that a cycle of pathogen colonization, bacteriophage infection, pathogen clearance, and re-colonization occurs on farm. Without understanding what relationship exists in nature between the bacteriophages, their pathogenic prey, the microbial ecosystem, and the host animal, we cannot hope to utilize this biological pathogen control system to its fullest to improve food safety.

It appears that the use of bacteriophages as a preharvest pathogen reduction strategy against *Salmonella* may be more complicated than previously considered. This difficulty may be due in large part to the observed relatively narrow spectra of phages isolated against the diversity of *Salmonella* serotypes. To reduce *Salmonella* in the U.S. swine population, *Salmonella*-killing phages that infect the serotypes most commonly involved in human illnesses and swine health issues must be obtained from multiple swine sources to reduce the likelihood of phage resistance and to increase the probability of treatment success.

Conclusions

Our results indicate that lytic bacteriophages are fairly widespread across commercial swine production facilities, but they may be present at relatively low populations. Phages capable of killing *Salmonella* Typhimurium are found in commercial swine, but not at a high prevalence. This is potentially due to a predator–prey cycle between the phages (predator) and *Salmonella* (prey) populations. These results suggest that because this cycle naturally exists in the commercial environment, phages 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 foodborne illness.

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Disclaimer

Proprietary or brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product, or exclusion of others that may be suitable.

Disclosure Statement

No competing financial interests exist.

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