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

Foodborne *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*,

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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 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 antisera poly A-1 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 (10⁶ 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 mm 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 FFFSU culture collection. Other bacterial species tested for bacteriophage activity included Salmonella derby, S. typhimurium, S. dublin, S. enteritidis, S. cholerasuis, S. montevideo, S. mbandaka, Enterococcus faecalis, Enter. 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.

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

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


