

Title: Enhanced virulence and treatment of multiple antibiotic resistant *Salmonella choleraesuis* in swine – NPB #07-076

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Industry Summary:

Previous studies revealed that protozoa (amoebae) can augment the virulence (ability to cause disease) of certain *Salmonella* strains that are resistant to multiple antibiotics. This phenomenon has not yet been observed in swine because many of these strains have low virulence in domestic pigs. However, resistance to multiple antibiotics was recently discovered in *Salmonella choleraesuis*, a strain that causes the most severe type of disease in swine. Enhancing virulence in *Salmonella choleraesuis*, would be devastating for the swine industry and this recent discovery is the basis for this project.

The first objective of this project was to evaluate the possibility that protozoa can augment the virulence of multiple antibiotic resistant *Salmonella choleraesuis*. The second objective was to determine the best antibiotic for treating this infection.

The research involved evaluating the ability of protozoa to enhance *Salmonella choleraesuis* in the laboratory and in swine. Laboratory studies involved measuring the ability of *Salmonella choleraesuis* to penetrate host cells, i.e. cellular penetration is an important part of *Salmonella* virulence, after exposure to protozoa. Swine studies involved orally infecting 10 day-old pigs with *Salmonella choleraesuis* exposed to protozoa. Swine were monitored for signs of disease and necropsies were performed in order to determine the amount of *Salmonella* in the animals. Some pigs were treated with either of two antibiotics: ceftiofur or amikacin.

Results from these studies revealed that protozoa are capable of enhancing the cellular penetration of *Salmonella choleraesuis* by approximately 700%. Animal studies revealed that protozoa-exposed *Salmonella*

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choleraesuis were capable of causing disease at 24 hours earlier compared to pigs infected with *Salmonella choleraesuis* that had not been exposed to protozoa. Tissue samples revealed that *Salmonella choleraesuis* was ten times more prevalent in swine infected with *Salmonella choleraesuis* exposed to protozoa. Both ceftiofur and amikacin ameliorated signs of disease (fever, diarrhea, and lethargy) although ceftiofur-treated pigs had a smaller load of *Salmonella choleraesuis*.

The results indicate that multiple antibiotic *Salmonella choleraesuis* can be more virulent after exposure to protozoa. Protozoa are water-borne common microbes and thus it appears that the combination of protozoa and *Salmonella choleraesuis* can lead to a dramatic course of *Salmonella* infection in swine. Ceftiofur seems to be the most appropriate treatment for this infection.

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Scientific Abstract:

Previous studies revealed that protozoa can augment the virulence of certain multiresistant *Salmonella* strains bearing the SGII integron. This phenomenon has not yet been observed in swine because many of these strains, including *S. typhimurium*, have low virulence in domestic pigs. However, SGII has now been detected in *Salmonella choleraesuis*, the swine-adapted serotype. This strain is henceforth designated as mr-*Salmonella choleraesuis*.

The first objective of this project was to evaluate the possibility that protozoa can augment the virulence of mr-*Salmonella choleraesuis* *in vitro* and *in vivo*. The second objective was to determine the best antibiotic for treating this infection.

In vitro studies involved assessing the host cell invasion of mr-*Salmonella choleraesuis* after exposure to protozoa. *In vivo* studies involved orally infecting 10 day-old pigs with mr-*Salmonella choleraesuis* exposed to protozoa. Swine were monitored for signs of disease and necropsies were performed in order to determine the amount of *Salmonella* in the animals. Some pigs were treated with either ceftiofur or amikacin.

Results from these studies revealed that protozoa are capable mediating hyperinvasion (700% of controls) in mr-*Salmonella choleraesuis*. Animal studies revealed that protozoa-exposed mr-*Salmonella choleraesuis* were capable of causing disease at 24 hours earlier compared to pigs infected with mr-*Salmonella choleraesuis* that had not been exposed to protozoa. Spleen samples revealed that mr-*Salmonella choleraesuis* was ten times more prevalent in swine infected with this strain following its exposure to protozoa. Both

ceftiofur and amikacin ameliorated signs of disease (fever, diarrhea, and lethargy) although ceftiofur-treated pigs had smaller burden of mr-*Salmonella choleraesuis*.

The results indicate that mr-*Salmonella choleraesuis* is more virulent after exposure to protozoa. Protozoa are water-borne common microbes and thus it appears that the combination of protozoa and mr-*Salmonella choleraesuis* can lead to a dramatic course of *Salmonella* infection in swine. Ceftiofur seems to be the most appropriate treatment for this infection.

Introduction:

Multiple antibiotic resistant *Salmonella* presents a great challenge to the swine industry. First, *Salmonella* costs the U.S. \$100-200 million per year. Second, antibiotic resistance in *Salmonella* is increasingly prevalent thus generating a pathogen that is difficult to treat. Third, certain strains of antibiotic resistant *Salmonella* are more virulent than antibiotic sensitive strains.

One specific multiresistant strain, designated as definitive type 104 (DT104), is the paradigm for hypervirulence. Specifically, animals infected with DT104 are 13 times more likely to die when compared to those infected with non-DT104 *Salmonella* (Evans and Davies 1996). Recently, Dr. Carlson's laboratory identified the basis for this phenomenon. We found that a genetic element associated with the DT104 integron, a large cluster of genomic DNA containing multiple antibiotic resistance elements and other genes unrelated to antibiotic resistance, upregulates virulence genes under certain conditions. These conditions are dependent upon the growth of DT104 inside protozoa (Rasmussen, Carlson et al. 2004), eukaryotic microbes that engulf bacteria as a food source. *Salmonella* is one of the few bacteria that can survive within protozoa.

A possible model for DT104 hypervirulence in swine begins with DT104 being engulfed by free-living protozoa such as amoebae. Free-living amoebae have been shown to harbor *Salmonella* and mediate hypervirulence in other pathogens (Cirillo, Falkow et al. 1994; Cirillo, Falkow et al. 1997). Amoebae, like DT104 or any *Salmonella*, are ubiquitous in the environment and are often associated with water. The most likely site for DT104-amoebae interactions would be in water contaminated with *Salmonella* by swine, birds, and reptiles such as turtles as we have described recently (Doling, Carlson et al. 2004). This would be of special concern for swine raised outside although the DT104-amoebae interaction is still very feasible for swine raised in confinement. Based on the relationship between amoeba in water-cooling units and the respiratory pathogen *Legionella*, it is also possible that hypervirulent *Salmonella* could be inhaled.

The model also has an intra-amoebae component in which the DT104 grows inside the amoebae and this harsh environment induces the activation of SO13, an uncharacterized gene in the DT104

integron. The protein encoded by SO13 then overactivates the expression of *hila*, a major promoter of *Salmonella* virulence. The abundance of HilA leads to the maximization of invasion, the ability to physically enter mammalian intestinal cells thus providing an access route to the systemic circulation, and thus the maximization of virulence since invasion is a major facet of salmonellosis.

Ingested DT104-loaded amoebae are translocated to the swine stomach where they are lysed thus liberating the hyperinvasive DT104. The pathogen then moves to the ileum where epithelial cells are invaded, at approximately 10 times the normal rate of invasion, leading to profuse diarrhea and systemic infection. This model has not been widely observed in swine since DT104 belongs to the Typhimurium serotype which does not usually cause significant disease in this host. However, this DT104 hypervirulence paradigm has recently become much more relevant. *Salmonella enterica* serotype Choleraesuis (*S. choleraesuis*), the strain of *Salmonella* that is adapted to swine, was recently shown to have acquired the DT104 integron. The DT104 integron is not specific to *S. typhimurium* since it has been observed in other serotypes such as *S. agona*, *S. infantis* (Carlson, Bolton et al. 1999), and *S. meleagridis*. The former two serotypes can exhibit the protozoa-mediated hyperinvasion observed in DT104 (Rasmussen, Carlson et al. 2004) while *S. meleagridis* has not yet been evaluated for this phenotype.

Extension of our hypervirulence model to *S. choleraesuis* begins with multiresistant *S. choleraesuis*, i.e. an isolate possessing the DT104 integron, being engulfed by an amoeba in an aqueous environment. The *S. choleraesuis* then survives in the amoeba and the virulence genes are hyperactivated as in the DT104 model. The amoeba is then ingested by a pig and is subsequently lysed in the stomach and the hypervirulent *S. choleraesuis* is liberated. *S. choleraesuis* can then invade the intestinal lining and cause a rapid systemic disease that is even worse than a conventional *S. choleraesuis* infection. That is, very high mortality with few antibiotics available for treatment. The hypervirulent *S. choleraesuis* could then be shed and infect other animals. This scenario could be devastating for the swine industry thus investigating this phenomenon is of great relevance.

Objectives:

(1) To evaluate the possibility that multiple antibiotic resistant *Salmonella enterica* serotype Choleraesuis (*S. choleraesuis*), the serotype of *Salmonella* that is adapted to swine, and *Salmonella enterica* serotype Typhimurium strain DT104 (DT104) can exhibit protozoa-mediated hypervirulence in swine.

(2) To find an appropriate treatment for this potentially devastating malady.

Materials & Methods:

Experiments were sequentially performed *in vitro* (Phase 1) and *in vivo* (Phase 2). The *in vitro* phase entailed tissue culture invasion assays and the *in vivo* phase involved infectivity experiments in baby pigs. Both phases relied upon immortalized cultures of *Acanthamoeba castellanii* (*A.c.*), a paradigm for free-living amoebae (Cirillo, Falkow et al. 1994; Cirillo, Falkow et al. 1997), while the *in vitro* phase also included certain Entodiniomorphid protozoa (EPz) found in mammals. EPz can mediate *Salmonella* hypervirulence in other species thus providing a positive control for the *in vitro* studies.

③ Phase 1: *In vitro* invasion assays.

Tissue culture invasion assays, a.k.a. gentamicin protection assays, are a commonly used tool for evaluating the pathogenicity of *Salmonella* (Gianella, Washington et al. 1973). In these assays, *Salmonella* strains are incubated with tissue culture cells such as HEp-2 cells. Extracellular, i.e. non-invasive bacteria, are then lysed using gentamicin. The HEp-2 cells are then lysed and the intracellular *Salmonella*, i.e. those that have invaded, are isolated on agar plates and then enumerated.

For these experiments, four strains were employed: multiresistant DT104 strain LNWI; antibiotic sensitive DT104 strain TH11 (Carlson, Bolton et al. 1999); multiresistant resistant *S. choleraesuis*; and antibiotic sensitive *S. choleraesuis* (as-*S.ch.*). Strain LNWI will be used as the DT104 model for EPz-mediated hyperinvasion. Invasion was assessed for each strain following recovery from either *A.c.* or EPz. Specifically, *A.c.* cultures were propagated and suspended in PYG media while EPz were isolated in Coleman's buffer D (Coleman and Reynolds 1982). *Salmonella* were then added to the protozoa, at a multiplicity of infection equal to 100, and the co-culture was incubated overnight. As negative controls, each strain was incubated with PYG media and Coleman's buffer D in the absence of protozoa. The protozoa (*A.c.* and EPz) were then lysed in a mini-beadbeater for 60 sec at 4,800 rpm using 2.5 mm glass beads in 1.5ml vials. Recovered *Salmonella* were enumerated and immediately used in a tissue culture invasion assay in which the pathogen was incubated with HEp-2 cells. Following the antibiotic protection and HEp-2 lysis phases, lysates were plated in triplicate on media containing antibiotics that select for specific *Salmonella* strains. The following day, *Salmonella* colonies were counted and invasion was quantitated. Hyperinvasion was determined by comparing invasion from *Salmonella* recovered from protozoa (principles) versus those exposed only to protozoa culture media (controls). An additional method for determining hyperinvasion was to compare invasion from *Salmonella* recovered from protozoa in multiresistant strains versus antibiotic sensitive isostrains. Statistical analysis included an analysis of variance with Scheffe's F test for multiple comparisons.

③Phase 2: *In vivo* experiments.

The four strains (DT104-LNWI, TH11, mr-*S.ch.*, and as-*S.ch.*) used in "Phase 1" were used in "Phase 2". These strains were incubated with *A.c.* and the resulting *A.c.-Salmonella* mixture was orally inoculated into baby pigs using five pigs per group. EPz studies were not be pursued since EPz are not

likely to infect swine *in vivo*. Control studies entailed inoculating pigs with HEp-2 cells loaded with one of the four strains. Four control groups, i.e. one per strain, plus four principal groups times six pigs per group required 48 pigs.

Following the infection, pigs were monitored every 6-8 hours for diarrhea, dehydration, and pyrexia. At 12 hours and every twelve hours thereafter, blood was drawn for the quantitative assessment of systemic *Salmonella*. Quantitation entailed plate counts with BGS plates containing the appropriate antibiotics. Swine were euthanized when they became recumbent, ataxic, pyrexic (rectal temperature greater than 106°F), or greater than 8% dehydrated. All remaining swine were euthanized at five days after infection. Hypervirulence was assessed by comparing colony-forming units (CFUs) for the antibiotic resistant strain versus its antibiotic sensitive isostrain and by comparing *A.c.*-dependent CFUs versus HEp-2-dependent CFUs for each strain.

An additional 12 pigs were used in treatment studies. Specifically, six pigs were inoculated with mr-*S.ch*-loaded *A.c.* and treated with long-acting ceftiofur (single IM dose of EXCEDE® (Pfizer), 5mg/kg) while another six pigs were inoculated similarly and treated with amikacin (25 mg/kg, IM, daily). Antibiotics were given at 36-48 hrs post-inoculation depending upon the observation of clinical signs. Pigs were euthanized after one week of treatment. As described above, blood was drawn and subjected to bacterial enumeration.

Results:

③Objective 1:

As shown in Table 1, multiresistant *S. choleraesuis* is more invasive upon exposure to host-associated protozoa. As shown in Table 2, multiresistant *S. choleraesuis* is more invasive upon exposure to free-living protozoa. These findings are consistent with our previous studies involving *Salmonella typhimurium*.

Strain	Growth condition	% Invasion
Antibiotic-sensitive <i>S. choleraesuis</i>	no protozoa	1.1 ± 0.2%
Multiresistant <i>S. choleraesuis</i>	no protozoa	1.4 ± 0.3%
Antibiotic-sensitive <i>S. choleraesuis</i>	host-associated protozoa	1.1 ± 0.2%
Multiresistant <i>S. choleraesuis</i>	host-associated protozoa	6.6 ± 0.5%

Table 1. Percent invasion of multiresistant *S. choleraesuis* exposed to host-associated protozoa.

Strain	Growth condition	% Invasion
Antibiotic-sensitive <i>S. choleraesuis</i>	no protozoa	0.9 ± 0.4%
Multiresistant <i>S. choleraesuis</i>	no protozoa	1 ± 0.2%
Antibiotic-sensitive <i>S. choleraesuis</i>	free-living protozoa	1.5 ± 0.3%
Multiresistant <i>S. choleraesuis</i>	free-living protozoa	7.1 ± 0.8%

Table 2. Percent invasion of multiresistant *S. choleraesuis* exposed to free-living protozoa.

③Objective 2: As shown in Fig. 1A, protozoa-exposed DT104 is hypervirulent in swine. Fig. 1B illustrates the same phenomenon for SGI1-bearing *S. choleraesuis* exposed to free-living protozoa while Fig. 1C compares the relative pathogen burdens for the two SGI1-bearing strains. Fig. 2 demonstrates that ceftiofur-treated swine have diminished pathogen loads of SGI1-bearing *S. choleraesuis* exposed to free-living protozoa.

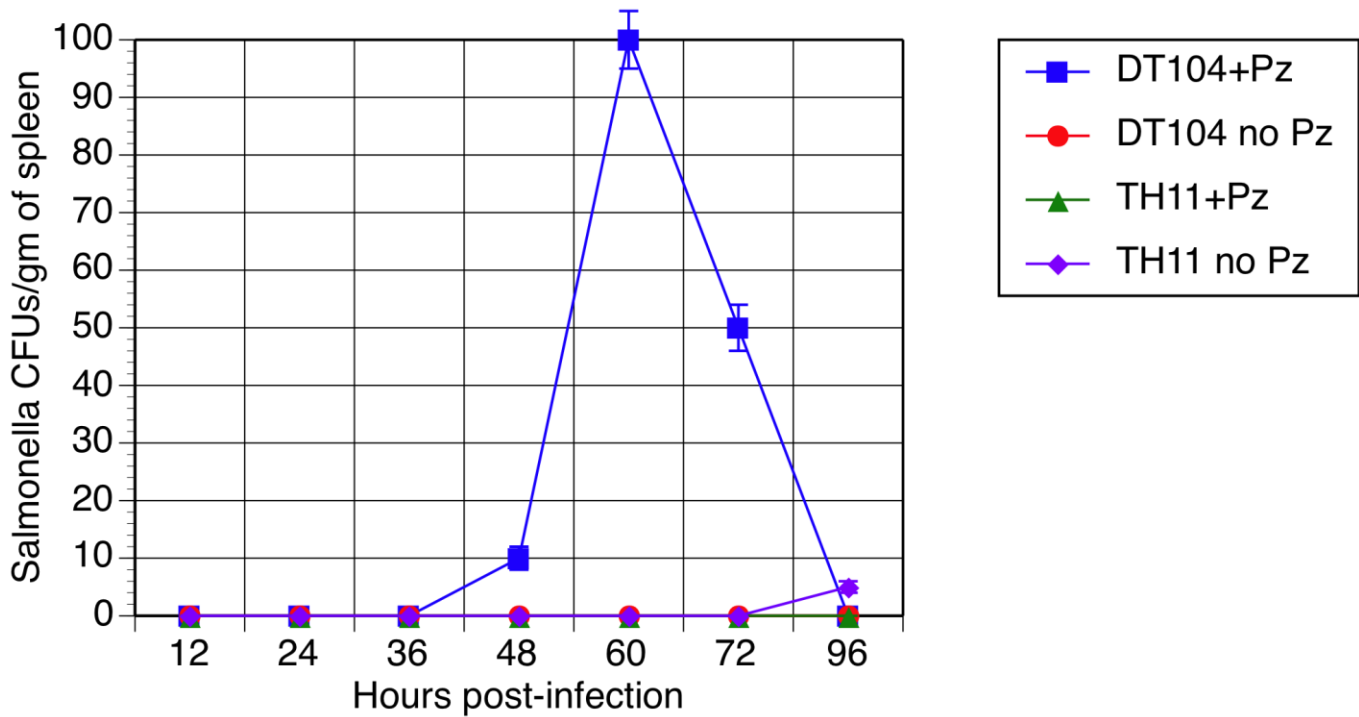


Fig. 1A: *Salmonella* recovery from swine infected with the multiresistant SGI1-bearing DT104 (DT104) or antibiotic sensitive DT104 (TH11). “+Pz” indicates infection with *Salmonella*-laden free-living protozoa (*Acanthamoebae*) while “no Pz” indicates infection with *Salmonella*-laden HEp-2 cells.

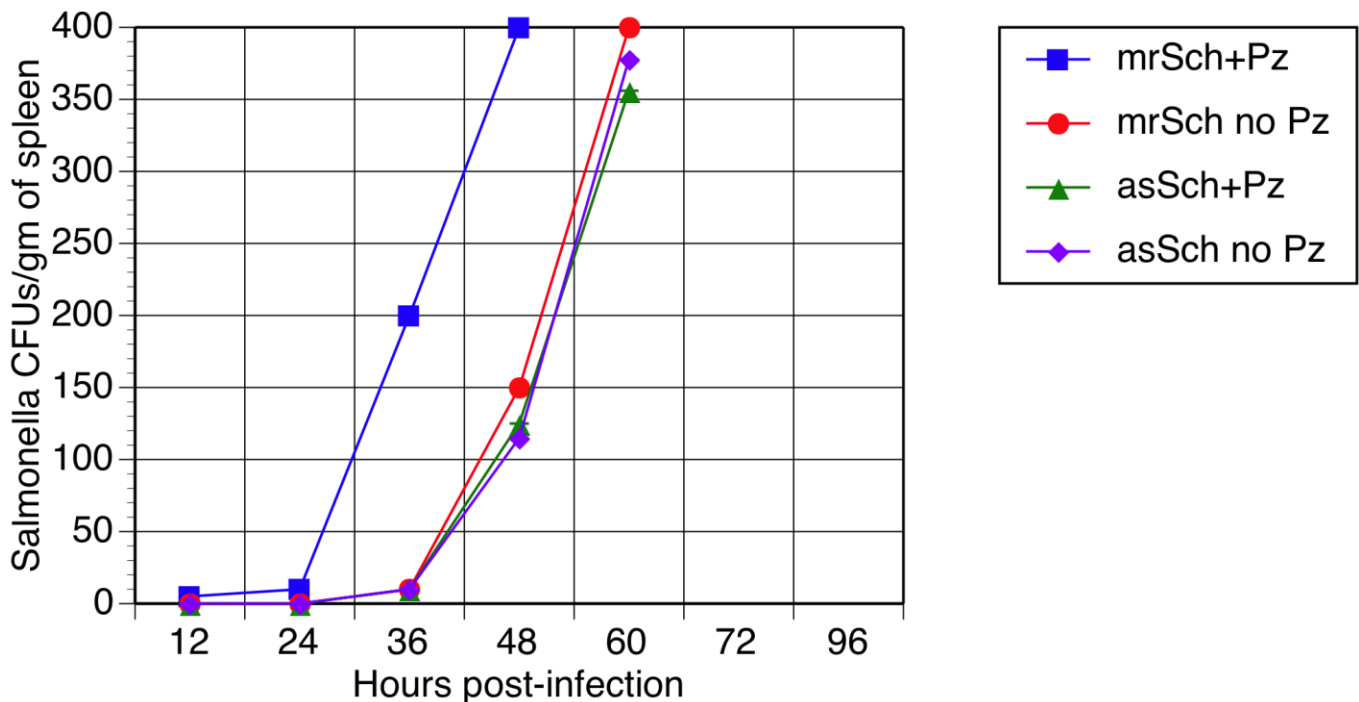


Fig. 1B: *Salmonella* recovery from swine infected with the multiresistant SGI1-bearing *S. choleraesuis* (mrSch) or antibiotic sensitive *S. choleraesuis* (asSch). “+Pz” indicates infection with *Salmonella*-laden free-living protozoa (*Acanthamoebae*) while “no Pz” indicates infection with *Salmonella*-laden HEp-2 cells. All swine were euthanized at or before 60 hours post-infection.



Fig. 1C: *Salmonella* recovery from swine infected with the multiresistant SGI1-bearing *S. choleraesuis* (mrSch) or multiresistant SGI1-bearing *S. typhimurium* (DT104). “+Pz” indicates infection with *Salmonella*-laden free-living protozoa (*Acanthamoebae*) while “no Pz” indicates infection with *Salmonella*-laden HEp-2 cells. All *S. choleraesuis*-infected swine were euthanized at or before 60 hours post-infection.

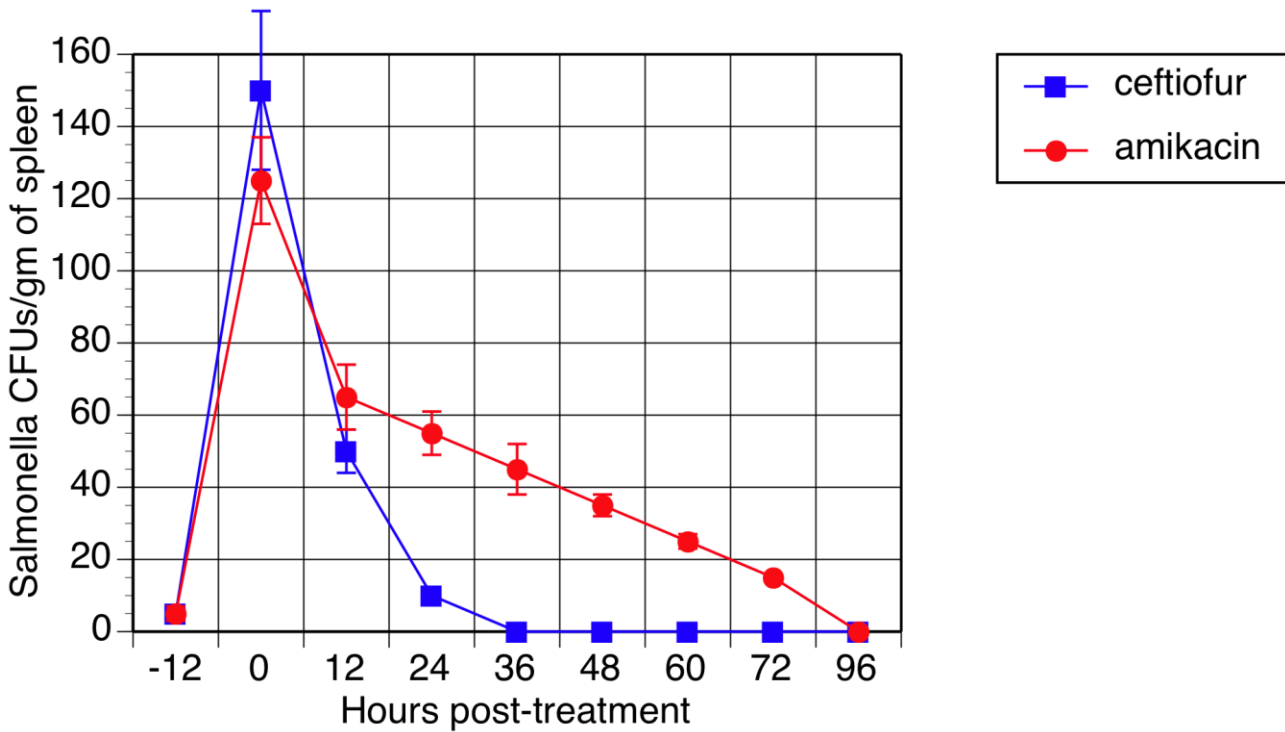


Fig. 2: *Salmonella* recovery from swine infected with the multiresistant SGI1-bearing *S. choleraesuis* (mrSch) exposed to protozoa. At the first signs of salmonellosis, swine were treated with either ceftiofur or amikacin.

Discussion:

Our findings indicate that free-living protozoa can exacerbate swine salmonellosis resulting from infection with SGI1-bearing *S. choleraesuis*. Exposure to free-living protozoa is an unknown entity but the swine industry must now be vigilant of this possibility. It appears that ingestion of *S. choleraesuis*-laden protozoa could result in a fast-progressing cases of salmonellosis with the potential for high mortality rates. Ceftiofur appears to be the best treatment option based on these findings. However, we did not examine enrofloxacin in this study since fluoroquinolones were banned for use in swine at the time of the *in vivo* experiments. Enrofloxacin is now approved for use in swine although ceftiofur would still be the preferred antimicrobial agent in juvenile swine since fluoroquinolones are chondrotoxic in immature patients.