Investigation of pathogenicity, competitive fitness, and novel methods for rapid diagnosis of *Salmonella* 4,[5],12:i:- in swine

by

Samantha Ann Naberhaus

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Program of Study Committee:
Amanda Kreuder, Co-major Professor
Adam Krull, Co-major Professor
Orhan Sahin

The study author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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ABSTRACT

Salmonella enterica subsp. *enterica* serovar 4,[5],12:i:- is widely accepted to be a monophasic variant of *Salmonella* Typhimurium. *Salmonella* 4,[5],12:i:- has been increasing significantly in prevalence worldwide over the past decade while *S.* Typhimurium has been subsequently decreasing. Both *Salmonella* serovars 4,[5],12:i:- and Typhimurium are contained in *Salmonella* serogroup B, which is currently the predominant serogroup in swine in the United States. However, this serogroup contains serovars of a wide range of pathogenic potential, with Typhimurium generally being associated with enterocolitis and Agona and Derby generally being considered to be of lesser pathogenicity. The pathogenic potential for *S.* 4,[5],12:i:- in swine is largely unknown but is hypothesized to be similar to *S.* Typhimurium based on genetic similarities. Current diagnostic procedures for the detection and identification of *Salmonella* have a wide range of sensitivities, and culture-based protocols have a prolonged turn-around time to serovar-level identification due to the complex serotyping process. To facilitate more rapid detection and serovar-level identification, the work conducted within this thesis validated a multiplex real-time PCR capable of detecting *Salmonella* spp. in general and differentiating *S.* 4,[5],12:i:- and *S.* Typhimurium from other lesser-pathogenic serovars. Further work was focused on determining the level of clinical disease, colonization of tissues, and persistence of infections to be expected with the *S.* 4,[5],12:i:- in swine. For this study, pigs were experimentally infected with *S.* 4,[5],12:i:-, *S.* Typhimurium, or *S.* Derby to compare *S.* 4,[5],12:i:- to the pathogenic *S.* Typhimurium and less-pathogenic *S.* Derby. This work demonstrated that *S.* 4,[5],12:i:- induces similar levels of clinical disease, tissue colonization, and persistent infections to that of *S.* Typhimurium, both of which were more severe than that of *S.*

Derby. Simultaneous infection of swine with equal amounts of *S.* 4,[5],12:i:- and *S.* Typhimurium revealed *S.* 4,[5],12:i:- is detected in higher levels in the feces, tonsils and ileocecal lymph nodes than *S.* Typhimurium, indicating a potential increased competitive fitness of the monophasic variant. The collective results of these studies provide improved diagnostics and needed insight into the disease causing ability of an increasingly prevalent serovar of *Salmonella* that is capable of infecting swine, humans, and other species.

CHAPTER 1:
GENERAL INTRODUCTION

Specific Aims and Significance

Salmonella 4,[5],12:i:- is considered a monophasic variant of *Salmonella* Typhimurium. However, even with the increasing prevalence of the monophasic serovar, much remains unknown regarding its potential as a pathogen of swine. Therefore, the goal of this work was to characterize *Salmonella* 4,[5],12:i:- as a pathogen of swine and validate rapid testing methods for *S.* 4,[5],12:i:- and *S.* Typhimurium. We hypothesized that *S.* 4,[5],12:i:- is a primary pathogen of swine similar to that of *S.* Typhimurium and has a greater fitness within the swine host. To test this hypothesis, four specific aims were developed: 1) document the prevalence of *S.* 4,[5],12:i:- in swine; 2) validate a multiplex real-time PCR to facilitate the rapid detection of *Salmonella* and differentiation of *S.* 4,[5],12:i:- and *S.* Typhimurium from other serovar of potentially lesser-pathogenicity; 3) characterize the disease, tissue colonization, and persistence of *Salmonella* 4,[5],12:i:- relative to better known serovars; and 4) evaluate the competitive fitness of *S.* 4,[5],12:i:- and *S.* Typhimurium following simultaneous inoculation of swine with both serovars.

Organization of Thesis

This thesis is organized in three chapters. The first chapter includes a literature review of pertinent peer reviewed studies related to the genus of *Salmonella* and the serovar *Salmonella* 4,[5],12:i:-. The second chapter describes the changes in prevalence of relevant serovars of *Salmonella* in swine and the validation of a real time PCR to facilitate rapid detection and identification of *Salmonella* 4,[5],12:i:- and Typhimurium. The third chapter describes three *in vivo* studies of swine conducted to evaluate the pathogenicity and competitive fitness of *Salmonella* 4,[5],12:i:- relative to other serovars of *Salmonella*.

Literature Review

General information on the *Salmonella* genus

The *Salmonella* genus is in the *Enterobacteriaceae* family, a family comprised of gram negative rods that are facultative anaerobes, non-spore forming, motile, catalase positive, and oxidase negative[1,2]. The *Salmonella* genus contains very resilient bacteria, making them able to survive in a variety of environments, including pH ranging from 4.0 to 9.5 and temperatures ranging from 8˚C to 45˚C[1]. Depending on the specific serovar and isolate, the survivability in the environment may extend outside of the listed ranges[1]. This hardiness facilitates its persistence in the environment, in livestock feed, and in human food. Salmonellosis (colonization of a *Salmonella* species in a host) occurs via the transmission of bacteria through the fecal-oral route[1,3,4]. The focus of this review will be on salmonellosis in swine and the impact on human health, although infections of other species will be mentioned briefly. Additionally, the focus will be placed on the newly emerging serovar, *Salmonella enterica* subspecies *enterica* serovar 4,[5],12:i:-.

Nomenclature and serotyping of *Salmonella*

The nomenclature system for the *Salmonella* genus contains many components. Starting with the species level, identified by genetic relation and biochemical testing, there are two species, *bongori* and *enterica*[1,5]. The species are then divided into subspecies based on biochemical testing, normal habitat, and genetic relation; species *enterica* includes subspecies I, II, IIIa, IIIb, IV, and VI while species *bongori* contains subspecies V[1,5]. *Salmonella enterica* subspecies I, also known as subspecies *enterica*, causes the majority of the *Salmonella* infections in warm blooded animals and humans[1] and will therefore be the focus of this paper. Subspecies

are then broken down into serogroups, which is based on the somatic, or "O", antigen. The most common serogroups involved in infections of warm blooded animals and humans are A, B, C1, C2, D, and E, although additional serogroups exist[5]. While identification of the serogroup is beneficial in gaining a better understanding of the potential pathogenicity of an isolate, it still leaves a wide range of potential serovars. The serogroups are divided into serovars (serotypes) based on their flagellar, or "H", antigens[5]. There are over 2,500 serovars with more being discovered to this day[6]. Serovars vary greatly in their pathogenicity, host range, and other characteristics. For example, within serogroup B are serovars Typhimurium, Agona, Derby, and Heidelberg. In a comparison of histologic lesions in swine associated with each of these serovars, 94% of samples from which *Salmonella* Typhimurium was isolated had lesions consistent with salmonellosis; this is in contrast to just 31% of samples from which *Salmonella* Agona, *Salmonella* Derby, or *Salmonella* Heidelberg were isolated that had salmonellosis-suggestive lesions[7]. Overall, *Salmonella* naming should be as follows: "*Salmonella* (*species*) subsp. (*subspecies*) (Serovar)". The serovar is named starting with the O antigens, then phase 1 flagellar antigens, and finally phase 2 flagellar antigens, all separated by colons[5]. For example, the common name of *Salmonella* Typhimurium, a serovar with potential to cause disease in all species, is actually *Salmonella enterica* subsp. *enterica* serovar 4,[5],12:i:1,2.

The conventional method for determining the serovar involves agglutination testing with multiple antisera targeting the somatic and flagellar antigens to identify the phenotypic characteristics, a process known as the Kauffmann-White-Le Minor Scheme[8]. In the United States as a part of the national surveillance program, the Kauffmann-White-Le Minor scheme is the chosen method for serotyping isolates from human specimens[6]. The challenge associated with this method revolves around the 64 potential O variants and 114 potential H variants,

thereby requiring a massive amount of antisera to maintain the ability to characterize all serovars[8]. More recently, other serotyping methods have evolved that focus more on the genetic rather than phenotypic characteristics, including pulsed field gel electrophoresis (PFGE), molecular serotyping by detection of the genes encoding the O and H antigens, and whole genome sequencing to name just a few[8]. Regardless of the method used to further characterize *Salmonella* isolates, identification at the serovar level is necessary to facilitate appropriate responses to the infection and to enable epidemiological studies.

Risk of salmonellosis to swine

Infections with *Salmonella* in swine, termed salmonellosis, are most common in weaned pigs approximately 6-12 weeks of age, but older and younger pigs can be infected in certain situations[4]. Morbidity, mortality, and clinical presentation of infection varies with the infecting serovar, age of pigs, and immunity possessed by the pigs[4]. Infection may present as either septicemia, which is more common with *Salmonella* Choleraesuis, or enterocolitis, which is more common with serovars such as *Salmonella* Typhimurium[4]. In the septicemic form, pigs become anorexic, lethargic, febrile, and dyspneic[4]. The enterocolitic form is generally associated with diarrhea, anorexia, lethargy, and fever[4]. The mortality of the septicemic form is typically higher than with the enterocolitic form while the opposite is often true for the morbidity of the two forms[4]. Gross lesions of both forms can include ulcerative and necrotic colitis and typhlitis, enlarged mesenteric lymph nodes, enlarged spleen and liver, and lung congestion[4]. The mandibular lymph nodes can be infected with *Salmonella* due to the oral route of infection, but they often appear normal grossly[9]. The variability in salmonellosis clinical signs, gross lesions, and distribution in the body complicates diagnosis of infection.

One of the more common and important forms of salmonellosis in swine is the asymptomatic infection. In this form, pigs shed *Salmonella* in their feces even though they lack clinical signs of disease[10]. While the pigs with subclinical salmonellosis do not have overt clinical disease, they do tend to be less productive than their uninfected counterparts as evidenced by a reduced average daily gain in pigs shedding higher levels of *Salmonella* in their feces[11]. The asymptomatic form is unfortunately complicated by the extensive and unpredictable variation in shedding patterns based on individual factors, environment, and *Salmonella*-strain specific factors. In one study, the shedding of *Salmonella* from naturally-infected pigs was followed from 10 weeks of age to the time of slaughter at 22 weeks of age[10]. The number of weeks in which the pigs were shedding ranged from just one week out of the 12-week period to nine weeks out of 12[10]. Even among those that shed *Salmonella* for the same amount of weeks, the specific weeks in which they were shedding varied by individual[10]. The variations in shedding made it clear that samples tested for *Salmonella* in the late finishing stage are not always an accurate reflection of the true *Salmonella* status of the animal tested as they may have not been shedding in the week or two prior to slaughter despite the continued presence of *Salmonella* in their system[10].

There is also some effect of serovar on the duration and pattern of shedding, but this needs explored further[10]. This variation emphasizes the need for rapid and accurate diagnostics on repeated samples from pigs to facilitate appropriate treatment prior to entering the slaughter facility. Others have examined the effect of dose and serovar on the shedding pattern of *Salmonella*[12]. The effect of the infecting dose appeared to be a more important factor than the serovar of the challenge when determining the level of shedding[12]. Swine infected with the same doses of *Salmonella* Yoruba, a generally non-pathogenic serovar found in feed, and *Salmonella*

Typhimurium, a classic pathogenic serovar of swine, shed *Salmonella* for a similar amount of time in their feces[12]. On the other hand, swine infected with 10^3 CFU and 10^6 CFU shed *Salmonella* for a significantly reduced duration when compared to those infected with 10^9 CFU, regardless of whether the pigs were infected with *Salmonella* Yoruba or *Salmonella* Typhimurium[12].

Salmonella, no matter the form of clinical disease, can colonize various tissues in an infected pig. These tissues can include but are not limited to the ileum and other parts of the intestinal tract, ileocecal lymph nodes, tonsils, and mandibular lymph nodes[9]. In randomly selected swine in a slaughter facility, the ileocecal lymph nodes were found positive for *Salmonella* 18.8% of the time, while 13.9% of ileal samples, 12.9% of mandibular lymph node samples, and 9.9% of tonsillar samples were positive for *Salmonella*[9]. Thirteen of 101 (12.9%) carcasses were positive for *Salmonella*, but 30% of those carcasses did not have a positive tissue sample from the corresponding pig which indicates the presence of cross-contamination between carcasses[9]. This underscores the added risk that infected animals present both from the initially infected carcass and the contamination of nearby carcasses in the slaughter facility[9]. The risk of *Salmonella* contamination must be reduced by starting at the farm level to decrease the occurrence of *Salmonella*-positive pigs entering slaughter facilities, which requires accurate and rapid diagnostic procedures.

Risk of salmonellosis to humans

The economic impact of reduced productivity and treatment costs in pigs with salmonellosis as well as the health impact of infections of swine and humans highlights the need to control *Salmonella* and eliminate the organism whenever possible. Non-typhoidal *Salmonella*

causes more than 1.2 million human illnesses worldwide and 100,000 human illnesses in the United States every year[13]; of these illnesses in the United States, approximately 40 deaths occur each year[14]. *Salmonella* is not the leading cause of foodborne illnesses, but it is still of primary concern given that it is the leading cause of foodborne illness-related hospitalizations and deaths in the United States[15,16]. Younger individuals, especially infants, tend to have the highest incidence of *Salmonella* infections[17,18].

Salmonella Typhimurium has commonly been implicated as a cause of food-borne illness in humans for many years. There was an outbreak reported in the United States in 2010 associated with pork[19], another outbreak in Denmark in 2011 associated with pork[19,20], and yet another outbreak in 2005 in Australia associated with lamb liver[21]. However, an increasing number of foodborne outbreaks of *Salmonella* 4,[5],12:i:- have also been reported in the recent years. Most commonly, these outbreaks are the result of *Salmonella* 4,[5],12:i:- contamination of pork products[22]. Specifically, one outbreak in France in 2011 was the result of contamination of pork, although the evaluation of raw materials and the food products did not test positive for *Salmonella*[22]. Another outbreak was reported in 2010 in France associated with beef consumption[23]. Yet another outbreak was reported in 2015 in the United States associated with pork consumption[24].

Prevalence by *Salmonella* serovar

In humans in the United States, the top five serovars in 2015 in order of most commonly involved in humans infections to least were Enteritidis, Typhimurium, Newport, Javiana, and 4,[5],12:i:-[18]. However, the prevalence of each serovar in any given host species does not remain static from year to year. For example, *Salmonella* Typhimurium has decreased in prevalence

from 2005-2015, for a total change of -26.2%[18]. On the other hand, *Salmonella* 4,[5],12:i:- has done the opposite and increased in prevalence from 2005-2015, for a total change of +194.2%[18]. Additionally, *Salmonella* 4,[5],12:i:- has increased in rank of serovar prevalence in human infections from 8th in 2005 to 5th in 2015[18]. It would be expected based on trends in livestock that the prevalence and rank of *Salmonella* 4,[5],12:i:- in human infections will continue to increase from 2015 to present.

Interestingly, *Salmonella* 4,[5],12:i:- was rarely reported prior to the 1990's[25]. The earliest report of the serovar was in 1946 when three isolates of *Salmonella* 4,[5],12:i:-, although identified as abnormal *Salmonella* Typhimurium at the time, were discovered as they failed to react in anti-i serum[26]. Between the under-reporting that likely occurs with salmonellosis in general and the potential misclassification of *Salmonella* 4,[5],12:i:-[25], it is difficult to determine exactly when *Salmonella* 4,[5],12:i:- actually emerged. What is clear is that in the past 10-15 years, there has been an increase in prevalence and recognition of *Salmonella* 4,[5],12:i:-.

In the European Union, a trend similar to that in the United States has been observed[17]. In order of most commonly involved in humans infections to least in Europe in 2016 were Enteritidis, Typhimurium, 4,[5],12:i:-, Infantis, and Newport[17]. *Salmonella* Typhimurium has been also been decreasing in prevalence in the EU since 2014 while 4,[5],12:i:- has been increasing although at a slower rate than that observed in the United States[17]. Based on zoonoses and food-borne outbreaks in 2016 in the European Union, interestingly, different livestock species contribute different serovars to humans infections[17]. Swine in the EU primarily contribute *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:- to human infections while cattle contribute *Salmonella* Typhimurium predominantly[17]. In contrast to the previously mentioned prevalence reports, in cases from 2001-2007 reported by national surveillance programs in the

European Union and United States, the World Health Organization (WHO) revealed that the top 20 most common serovars in humans in the European Union, Africa, Australia, and New Zealand did not include *Salmonella* 4,[5],12:i:-[27]. *Salmonella* 4,[5],12:i:- was likely not among the most prevalent serovars at this time because this study pre-dates the rapid increase of identification observed in *Salmonella* 4,[5],12:i:- over the last decade.

In humans in China from 2009-2010, *Salmonella* Typhimurium remained the most prevalent isolate, comprising 45.2% of the isolates, while *Salmonella* Enteritidis, *Salmonella* Stanley, and *Salmonella* 4,[5],12:i:- trailed behind with 12.5%, 7.1%, and 4.5% respectively[28]. Changes have likely occurred in the distribution of serovars since this study was conducted nearly a decade ago. From 2007-2011 in Switzerland, the prevalence of *Salmonella* 4,[5],12:i:- changed significantly, from making up just 5.8% of the *Salmonella* isolates from humans in 2007 to 18.2% in 2011[29]. It is important to keep in mind that the prevalence of serovars clearly varies by geographic location, but with all of the travel and trade that occur throughout the world pathogens have the potential to spread rapidly across national borders[27].

In swine, just like in humans, the common serovars vary based on geographic location. Based on samples submitted to the Minnesota Veterinary Diagnostic Laboratory (VDL) in 2014, swine were most commonly infected with, in order of highest to lowest prevalence: Agona, Typhimurium var 5- (Copenhagen), 4,[5],12:i:-, Derby, and Typhimurium[30]. *Salmonella* 4,[5],12:i:-, Agona, and Typhimurium all increased in prevalence from 2013-2014 while the remaining two serovars decreased in prevalence[30]. The data from the Minnesota VDL separated *Salmonella* Typhimurium and *Salmonella* Typhimurium var 5- into two serovar groups; however, there have not been any reports of differences in var 5+ and var 5- strains with respect to their disease-causing ability and are kept in the same serovar group in all other data sets.

Based on samples submitted to the Iowa State University VDL (ISU-VDL) from 2003-2015, the proportion of *Salmonella* serovars Typhimurium, Derby, and Heidelberg have all decreased, in contrast to serovars 4,[5],12:i:-, Infantis, and Johannesburg which have all increased[31]. The most dramatic increase was observed with *Salmonella* 4,[5],12:i:-, though, from 0% in 2003 to 30% of the swine *Salmonella* isolates in 2015[31].

In a study examining pigs in a slaughter facility in Portugal from 2003-2004, the serovars with the highest prevalence were Typhimurium and Rissen[9]. *Salmonella* 4,[5],12:i:- was isolated in just 4.4% of samples[9]. In a Canadian study conducted during an unknown time period following naturally infected pigs from 10-weeks until slaughter at 22 weeks of age, the most prevalent serovars shed in their feces were Typhimurium, Livingstone, Senftenberg, and Infantis[10]. Furthermore, the more common serovars involved in infection was different for the initial infection of pigs in the study, in which *Salmonella* Senftenberg and *Salmonella* Typhimurium were the most common, and re-infection at a later point in the study, in which *Salmonella* Livingstone was the most common[10]. The authors of this study proposed that re-infection with a different serovar from what that pig was initially infected with may be due to a lack of cross-protection between the serovars of *Salmonella*[10].

Diagnostic testing for *Salmonella*

Detection and identification methods for *Salmonella* are highly variable in their process, sensitivity, and specificity. Aside from differences in enrichment broths, selective agars, and timing of incubation of bacteriological culture methods, there are serologic tests and genetic tests available with the same variation in sensitivity and specificity. The International Organization for Standardization (ISO), an organization to which the United States is a member, provides

specifications for products, services, and systems in an effort to ensure the quality, safety, and efficiency of many products and procedures worldwide[32]. The current ISO procedure for *Salmonella* identification from the food chain via culture is as follows: pre-enrichment of sample in buffered peptone water (BPW) for 18 hours at 37˚C, transfer from BPW to Rappaport-Vassiliadis (RV) broth or modified semisolid RV (MSRV) agar then incubate for 24 hours at 41.5˚C while simultaneously transferring from BPW to Muller-Kauffmann Tetrathionate-novobiocin (MKTTn) broth then incubating for 24 hours at 37˚C, then plate from selective enrichment to Xylose Lysine Deoxycholate (XLD) agar and an additional isolation agar and incubate for 24 hours at 37˚C, and finally test at least one suspect colony for confirmation as *Salmonella* species[33]. Testing only one suspect colony leaves a high risk of overlooking a less predominant serovar of *Salmonella* in a mixed infection of more than one serovar of *Salmonella*. Complete characterization to the serovar level is typically completed for only one colony per sample submitted to the majority of veterinary diagnostic laboratories as well, including the ISU-VDL.

Previous work has compared the sensitivity and specificity of five different culture methods for the isolation of *Salmonella* spp. from the feces of swine[34]. The methods, highly variable in terms of the media used and incubation times and temperatures, were as follows: 1) gram-negative (GN) broth→RV broth→xylose-lysine-tergitol-4 (XLT4) agar, each step incubated at 37˚C for 24 hours; 2) tetrathionate broth (TTB)→RV broth→XLT4 agar, all incubated at 37˚C, TTB incubated for 48 hours, RV broth and XLT4 agar incubated for 24 hours; 3) peptone broth→RV broth→XLT4 agar, all incubated for 24 hours, peptone broth and XLT4 incubated at 37˚C, RV broth incubated at 42˚C; 4) peptone broth→RV plate, all incubated for 24 hours, peptone broth incubated at 37˚C, RV plate incubated at 42˚C; 5) peptone

broth→TTB→XLT4 agar, all incubated for 24 hours, peptone broth and XLT4 agar incubated at 37 degrees C, TTB incubated at 42˚C[34]. The highest sensitivity of any one method from swine fecal samples was 91.3% with method 2, while the other methods ranged from 0-80.4%[34]. Utilizing two of the methods simultaneously resulted in up to 100% sensitivity[34]. Unfortunately, it is not feasible for most diagnostic laboratories to use two methods for diagnosis of salmonellosis due to increased costs, time, and labor. In another study comparing different culture methods as well as PCR and ELISA testing, it was concluded that while the specificity was outstanding for all testing methods, ranging from 0.99-1, the sensitivity was much more variable, ranging from 0.23 with the OPTIMA ELISA test to 0.98 with the modified semi-solid Rappaport Vassiliadis (MSRV) agar and MSRV with PCR method[35]. The MSRV methods began with BPW as a non-selective enrichment broth, then transferred to MSRV agar, then either subcultured to XLD agar and brilliant green agar (BGA) or screened on the PCR[35]. Thus, it appears that while *Salmonella* diagnostics have an extremely low risk of false positives, there is a high risk of false negatives.

Quantification of *Salmonella* in a sample can be an extremely useful measure for veterinarians and producers as it aids in describing the risk of herd-level spread of the organism[12]. Unfortunately, quantification presents unique difficulties. Differentiation of *Salmonella* from other fecal organisms poses a challenge even on *Salmonella*-selective agar[12]. Also, low numbers of organisms may be shed in feces, which further complicates enumeration of the bacteria. An enrichment step is often utilized to mitigate this problem which eliminates the ability to truly quantify the amount of *Salmonella* present in a sample[12].

For serologic testing, the serovar-specific antibody response must be known in order to interpret the results. In another study, pigs infected with low levels of *Salmonella* Typhimurium

and *Salmonella* Yoruba failed to exceed the threshold of the assay detecting anti-*Salmonella* antibodies at all sample collection time points, while pigs infected with a high level of *Salmonella* Typhimurium and *Salmonella* Yoruba had a peak seroprevalence by 14 days post infection (DPI) and 28 DPI, respectively[12]. In pigs infected with *Salmonella* Typhimurium, *Salmonella* Livingstone, or *Salmonella* Senftenberg, seroconversion occurred four weeks after the shedding of *Salmonella* in feces peaked[10]. In a different set of nursery pigs, sourced from known high-*Salmonella* prevalence farms and followed until slaughter, the serologic response to *Salmonella* Typhimurium infection peaked at 17 weeks of age, eight weeks after the peak fecal *Salmonella* shedding[36]. With the delay in antibody response relative to detectable levels of *Salmonella* in feces, serologic testing may be useful for determining previous *Salmonella* exposure in an individual animal that is culture-negative for *Salmonella*. Serology also overcomes the limitations of culture created by the variable shedding patterns, but only in the later stages of infection when seroconversion has occurred. Utilization of serologic and culture methods simultaneously may provide a better understanding of the overall current *Salmonella*-status and history of an individual pig.

Most recently, polymerase chain reaction (PCR) methods have been validated for *Salmonella* detection. These methods offer many advantages over culture, including reduced turnaround time, reduced subjectivity in reading tests, increased quantification ability, and potentially increased sensitivity and specificity. In one PCR assay targeting the *invA* gene, a gene common to all invasive *Salmonella* strains, to detect *Salmonella* spp. from equine feces, the specificity was 100% and the limit of detection was just 3-10 *invA* gene copies/μL DNA depending on whether or not enrichment broth was utilized[37]. A similar PCR assay was also validated to detect *invA*, and it was able to detect 67 *Salmonella*-positive samples while culture

detected merely 28, a sensitivity difference of 48-56% for culture and 87-95% for PCR (range determined by the Bayesian model assumptions applied)[38]. Although sensitivity was higher with PCR, the specificity of the PCR was less than 93% while culture was 100%[38]. In another quantitative real time PCR assay validated to differentiate *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Salmonella* 4,[5],12:i:-, there were four sets of primers and probes targeting *safA* for *Salmonella* Enteritidis identification, *fliA*, *fljB-hin*, and *hin-iroB* for *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:- identification[39]. The limit of detection of this assay was 3-14 gene copies (averaged across all gene targets)/PCR assay, although it varied slightly by each gene target[39]. In comparison of identification of serovar by PCR and culture, *Salmonella* Enteritidis matched for 99.0% of isolates while *Salmonella* Typhimurium and its variants matched for 98.2% of isolates[39]. The specificity of the PCR was 98%[39]. Other PCR tests have also been validated with the goal of detecting *Salmonella* in general or identifying specific serovars of *Salmonella*[40-42].

Selecting the appropriate sample type to maximize the efficacy of the diagnostic test is also critical and requires a balance between the ideal sample type and the sample type that can be reasonably collected. When comparing individual and pooled fecal samples from immature and adult cattle and swine, pooled fecal samples were better able to detect *Salmonella* from all groups except for adult, outdoor swine[43]. These findings are promising given that pooled fecal samples collected from the ground or floor are easier and more convenient to collect compared to individual fecal samples collected as the pig defecates or from the rectum. However, it is important to note that pooled fecal samples are obviously unable to determine the *Salmonella* status of each individual, which may be a problem if the goal is to treat only infected pigs rather than mass medication. Other sample types, aside from feces, are also available and include

environmental samples, oral fluids, and blood. Overall, the lack of standardization of *Salmonella* detection methods and the high level of variability between methods makes comparison of results from different laboratories and studies challenging. Further research to find more reliable methods of detection and quantification of *Salmonella* as well as the corresponding serovar-specific antibody response would aid in the interpretation of *Salmonella* detection data obtained from various sources.

Treatment of salmonellosis

Antimicrobials are available for the treatment of *Salmonella* spp. infections in swine. In a study of the risks associated with *Salmonella* shedding from swine at the slaughter facility, the median survival time of *Salmonella* spp. within the host was reduced by up to 60% with treatment[44]. However, this study did not specify what treatment protocol was followed by the farms included in the study, only stating that the treatment typically included antimicrobial drugs. The United States Food and Drug Administration Green Book, a listing of all approved animal drug products that is updated monthly, lists drugs included in the antimicrobial classes of penicillins, cephems, sulfonamides, and tetracyclines as effective in the treatment and control of bacterial enteritis caused by *Salmonella*[45]. The most definitive way to select an antimicrobial for treatment of salmonellosis is to perform *in vitro* culture with subsequent antimicrobial susceptibility testing. However, the Clinical and Laboratory Standards Institute (CLSI) guidelines lack breakpoints specific to many drug-bacteria combinations in veterinary medicine, and therefore antimicrobial susceptibility testing of *Salmonella* isolates requires interpretation based on breakpoints in human medicine or for other bacteria. Prior knowledge and experience with a herd undergoing an outbreak of *Salmonella* in addition to an understanding of the typical

antibiograms of the likely infecting serovar can be used to initiate an appropriate treatment without antimicrobial susceptibility testing results[46]. *Salmonella* 4,5,12:i:- is often resistant to ampicillin, streptomycin, sulfisoxazole, and tetracycline, with 50% or more of isolates being resistant to each of those antibiotics[47]. *Salmonella* Typhimurium has a similar resistance profile, but is much less commonly resistant to those antibiotics, with only 25% or less of isolates being resistant[47].

Based on several research studies, there are some antimicrobials to potentially avoid given the reported increased virulence of *Salmonella* Typhimurium, and presumably its monophasic variant, in the host during treatment. One study demonstrated that tetracycline treatment of multidrug resistant (MDR) *Salmonella* Typhimurium led to up-regulated expression of three virulence genes although this did not necessarily correlate to increased host cell invasion[48]. All of the *Salmonella* Typhimurium isolates utilized in this study possessed one or more genes that are known to confer resistance to tetracyclines, which is not common among *Salmonella* Typhimurium[13]. Another study that looked at *Salmonella* Typhimurium treatment with chlorotetracycline showed that pigs treated with chlortetracycline had significantly higher levels of *Salmonella* in their feces on DPI 2 and in tonsils at necropsy on DPI 7[49]. These findings are concerning given the potential for carcass contamination and subsequently pork product contamination as discussed previously. However, no other tissues, including distal ileum, ileocecal lymph nodes, cecal contents, and cecal mucosa, had an increased level of *Salmonella* when compared to non-chlortetracycline treated pigs[49]. Another study echoed those findings but determined that pigs treated with chlortetracycline shed higher levels of *Salmonella* Typhimurium up until DPI 7, rather than just on DPI 2[50]. In a group of pigs treated with sub-inhibitory concentrations of chloramphenicol, tetracycline, ampicillin, and streptomycin, both

chloramphenicol and tetracycline treatment increased the invasion abilities of *Salmonella* Typhimurium while ampicillin and streptomycin did not affect invasion[51]. Moreover, chloramphenicol and tetracycline reduced the expression of genes associated with motility of *Salmonella* while increasing the expression of genes associated with attachment and intracellular survival[51]. In addition to the aforementioned risks associated with certain antimicrobial treatments, there is also the increasing concern of development of and selection for antimicrobial resistant bacteria. It is well known that excessive use of antimicrobials promotes selection of antimicrobial resistance in the population of bacteria exposed to the drug[52], therefore, it is of interest to reduce the overall impact of treatment of salmonellosis by utilizing alternatives that may have lower risks.

With the risks associated with antimicrobial treatment, alternative management options for the treatment and control of *Salmonella* should be considered. Alternatives to antimicrobials can include heavy metals, such as zinc oxide or copper, and diet acidifiers. Zinc oxide added to the diet of pigs has been shown to be effective in improving average daily gain of four-week old weaned pigs when exposed to enterotoxigenic *E. coli*[53]. It also was able to increase the daily feed intake of the pigs and the gain-to-feed ratio[53]. Supplementation with zinc oxide also reduced the coliforms detectable in the ileum and colon while simultaneously increasing the ratio of lactic acid bacteria to coliforms in the intestines, indicating a positive effect on the gut microbiota[53]. Zinc oxide may also promote improved intestinal morphology, which may be beneficial in reducing the incidence and/or severity of enteric infections[53,54]. Copper can also be added to the diet and boasts the same potential outcomes as adding zinc to the diet[54]. Diet acidifiers show potential as antibiotic alternatives as they help to foster a gastrointestinal environment that promotes the growth of beneficial bacteria while potentially inhibiting growth of pathogenic

bacteria[54]. However, there are many different types of acids, including organic acids, inorganic acids, and acid salts, which may not have the same positive effects on pigs in general and especially on pigs exposed to *Salmonella*[54]. The simultaneous addition of organic acids, phytochemicals, and a permeablizing complex to the diet of pigs reduced the fecal shedding of *Salmonella* 4,[5],12:i:- at weeks 3, 6, and 8[55]. However, this combination of feed additives did not show the same beneficial effect on the average daily gain, feed conversion ratio, or daily feed intake[55]. With this being a relatively new concept for bacterial control, it is not surprising that more research must be done to determine the exact effects of various acids and metals to ensure the desired outcome is achievable.

***Salmonella* 4,[5],12:i:-**

Origin

With over 2500 serovars of *Salmonella* and more serovars still being discovered or emerging, some serovars have a well-understood pathogenesis, virulence, host species range, and general characteristics while others are severely lacking in this critical information. Many questions remain unanswered about the monophasic *Salmonella* 4,[5],12:i:-, including the cause of its emergence, the preferred host species, and resulting disease. Monophasic serovars of *Salmonella* in general could have originated from two main sources: 1) ancestral serovar that never developed the necessary mechanism to express a second flagellar phase or to switch from phase 1 to phase 2 flagella, or 2) mutation from a diphasic serovar[56]. Beginning with the antigenic formula, 4,[5],12:i:-, it is evident that this serovar belongs to serogroup B *Salmonella* which share common somatic antigens 4 and 12[57]. *Salmonella* Lagos (4,[5],12:i:1,5) and

Salmonella Typhimurium (4,[5],12:i:1,2) are the only two serovars that possess the same somatic and phase 1 flagellar antigens[57,58].

Based on the higher prevalence of *Salmonella* Typhimurium in comparison to *Salmonella* Lagos, the two most likely serovars from which this monophasic serovar was derived, it is more likely that *Salmonella* 4,[5],12:i:- originated from *Salmonella* Typhimurium[58]. An insertion sequence, IS200, was identified in *Salmonella* and *Escherichia coli* and is located in the intergenic region of *fliA* and *fliB*[59]. In the comparison of different serovars, *Salmonella* Typhimurium isolates possessed an amplicon of 1135 base pairs (bp) long while other serovars examined had only 424 bp long amplicons[59]. In alignment with the thought that *Salmonella* 4,[5],12:i:- originated from *Salmonella* Typhimurium, *Salmonella* 4,[5],12:i:- has been shown to possess a 1000 bp *fliA-fliB* intergenic region similar to that reported in *Salmonella* Typhimurium and in contrast to that of other serovars, including Lagos, Agona, Derby, and others which have 250 bp long intergenic regions[58,60]. Although there are slight inter-study differences in the length of the intergenic region of *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:-, the conclusion that each study reached is the same: *Salmonella* 4,[5],12:i:- possesses an IS200 sequence which closely resembles that of *Salmonella* Typhimurium.

Pulsed-field gel electrophoresis (PFGE)

PFGE can be utilized to compare the degree of relation between isolates, including isolates of different serovars as well as of the same serovar. When evaluating isolates of *Salmonella* from China, PFGE completed using enzyme *XbaI* revealed that 30 *Salmonella* Typhimurium isolates were identical to five *Salmonella* 4,[5],12:i:- isolates, signifying a possible relation between the two serovars[28]. Characterization of *Salmonella* 4,[5],12:i:- from Switzerland

by PFGE using *XbaI* showed that, of the 758 *Salmonella* 4,[5],12:i:- isolates evaluated, there were more than 150 different pulsotypes but only seven dominant pulsotypes contained the majority (66%) of the isolates[29]. This indicates that there is likely a close ancestral relation of these isolates. PFGE combining the profiles created by both *XbaI* and *BlnI* restriction enzymes resulted in at least 85% similarity among 69% of the tested *Salmonella* 4,[5],12:i:- isolates[61]. This also indicates that there is likely a close common ancestor of at least those isolates, however, greater than 31% of the isolates did not share that level of similarity and may have arisen from a less closely-related ancestor[61].

Multiple-locus variable analysis (MLVA)

While serotyping is a common method applied in the United States for subtyping of *Salmonella* to aid in determination of the source in epidemiological studies, phage typing, antimicrobial resistance profiles, and multiple-locus variable analysis (MLVA) are among some of the other options available and applied in other countries[62]. MLVA has some challenges when it comes to long-term surveillance due to evolution of the variable number tandem repeats (VNTR) that are targeted by MLVA, but there is a place for MLVA in tracing outbreaks given the high level of discrimination and reproducibility it provides[62]. In a comparison of 411 *Salmonella* 4,[5],12:i:- and 182 *Salmonella* Typhimurium isolates from Italy using MLVA, the isolates were broken into seven clusters, five of which contained isolates from both serovars[63]. Given the serovar specificity of MLVA, this further substantiates the hypothesis that *Salmonella* 4,[5],12:i:- is a variant of *Salmonella* Typhimurium[62,63]. The relatively low variability in MLVA profiles of *Salmonella* 4,[5],12:i:- isolates in comparison to *Salmonella* Typhimurium isolates

increases the likelihood that *Salmonella* 4,[5],12:i:- emerged much more recently, which is not surprising given the serovar prevalence changes over the recent years as discussed previously[63].

Some MLVA profiles of human-origin isolates have been shown to be very similar or the same as isolates from food producing animals, indicating a potential spread from one to the other[63]. This finding was supported by the results of another study evaluating isolates from Belgium which reported some profiles of isolates from humans were identical to those of livestock[64]. When comparing *Salmonella* 4,[5],12:i:- isolates from France using MLVA, there was significant overlap in MLVA type and the source, meaning that each MLVA type was found in the live pig, pork products, and humans, indicating a spread from one to the other[65].

Phage typing

Phage typing is based on the ability of certain bacteriophages to infect an isolate of *Salmonella*, determined by the presence of appropriate receptors on the bacterial cell wall and genetic characteristics of the bacteriophage[66]. The phage type of an isolate is reported as the bacteriophage or bacteriophages that are able to lyse the *Salmonella*[66]. Further evaluation of 40 4,[5],12:i:- isolates from Switzerland through phage typing showed that 55% of them were DT193, 15% were DT104b, 10% were DT120, and 2.5% were DT7, with the remaining isolates unable to be typed[29]. All of these phage types have been reported in *Salmonella* Typhimurium. Other studies comparing the two serovars to one another reached similar conclusions, with both *Salmonella* 4,[5],12:i:- and *Salmonella* Typhimurium isolates having phage types U302, DT120, and DT193[27,65]. Another study looking at the origin of *Salmonella* 4,[5],12:i:- determined that the majority (70%) of evaluated *Salmonella* 4,[5],12:i:- isolates are phage type U302[61]. In an evaluation of isolates from Korea, the most common phage types are DT193, DT104b, and

DT208, all of which are shared with *Salmonella* Typhimurium[67]. When evaluating phage types in different serovars of *Salmonella*, it was determined that a genomic region was found only in DT104 and DT U302 *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:- isolates but absent in *Salmonella* Lagos, providing further evidence that *Salmonella* 4,[5],12:i:- arose from *Salmonella* Typhimurium rather than *Salmonella* Lagos[68]. Phage types, or definitive types, are highly specific to even the serovar level[69,70], so the commonality between *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:- phage types provides additional evidence that *Salmonella* 4,[5],12:i:- originated from *Salmonella* Typhimurium.

Whole-genome sequencing (WGS)

Whole-genome sequencing has recently become a more widely used genotyping method due to improved affordability and accessibility[71]. This method provides a higher resolution of closely related isolates than other genotyping methods[71]. Although whole-genome sequencing has not been widely utilized for characterization of bacterial isolates, a few studies have been published comparing *Salmonella* 4,[5],12:i:- to *Salmonella* Typhimurium. In one study, it was determined that *Salmonella* 4,[5],12:i:- isolates from swine the Midwestern United States had a high level of similarity with *Salmonella* Typhimurium isolates from Europe[72]. Another study found that, of the 32 total *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:- isolates analyzed, *Salmonella* Typhimurium isolates were closely related to *Salmonella* 4,[5],12:i:- isolates[73].

Polymerase chain reaction (PCR)

In the study of Belgium isolates, based on PCR assays in accordance with the recommendations of the European Food Safety Authority (EFSA), 194 of the 253 *Salmonella* 4,[5],12:i:- isolates were confirmed to be monophasic variants of *Salmonella* Typhimurium with an absence of *fljB* amplification and presence of IS200 element[74]. The remaining Belgium isolates, 59 of 253, although they phenotypically presented as monophasic isolates, had maintained a biphasic molecular status[74]. Similar findings were reported from another study of monophasic isolates with 18.9% of these isolates being positive for the portion for the *fljB* gene targeted by the PCR primers, even though they were phenotypically monophasic[75].

In a comparison of *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:- isolates as well as a comparison of *Salmonella* 4,[5],12:i:- isolates from the United States, Spain, and Thailand, differences were revealed in the presence or absence of typical flagella-related genes based on location of origin and serovar[76]. Primers for this comparison included three that are considered highly specific for *Salmonella* Typhimurium and four others that were involved in the phase 2 flagellin expression, including *fliA, fljB, hin*, and *iroB*[68,76]. The Spain and Thailand-origin *Salmonella* 4,[5],12:i:- isolates were positive for two of the three *Salmonella* Typhimurium specific targets while the US-origin *Salmonella* 4,[5],12:i:- isolates were positive for only one of the three[76]. Additionally, the Spain-origin isolates were negative for all four targets related to phase 2 flagella while the US and Thailand-origin isolates were negative for all four targets except for *iroB*[76]. Another study compared a PCR assay that detects three *Salmonella* Typhimurium-specific regions and had been successfully tested against 117 other serovars of *Salmonella* to ensure a lack of cross-reactivity; the only serovar aside from *Salmonella* Typhimurium that tested positive for those three regions was *Salmonella* 4,[5],12:i:-[60].

Monophasic phenotype

Flagellar phase variation in *Salmonella* is a complex process, requiring the expression and functionality of multiple genes. In biphasic *Salmonella* serovars, the phase 1 flagellin is encoded by *fliC* while the phase 2 flagellin is encoded by *fljB*[77]. Only one flagellar phase is expressed at any one time, so there is a process by which the expressed flagellin is switched from phase 1 to phase 2 and phase 2 to phase 1. In a wild-type *Salmonella* Typhimurium, the amount of switching from phase variation from *fljB* to *fliC* and vice versa *in vitro* were 0.036 and 0.011 colonies switched per colonies tested, respectively, with similar rates of phase variation obtained from Peyer's patches and spleen of mice inoculated with *Salmonella* Typhimurium[78]. In a genomic region adjacent to *fljA* and *fljB*, known as the H segment, the *fljAB* promoter and *hin* gene are located[77,79]. The *hin gene* encodes a recombinase, which works in conjunction with the *fis* gene, *HU* gene, and host factors to regulate the reversible recombination of *hixL* and *hixR*, the regions flanking *hin*[80-82]. As recombination occurs at *hixL* and *hixR*, one of two outcomes occurs based on its orientation: 1) *fljAB* promoter is transcribed to cause *fljB* expression and *fljA* expression with subsequent *fliC* inhibition; or 2) *fljAB* promoter is not transcribed and subsequently *fljA* and *fljB* are not expressed, leading to *fliC* expression[79-81].

The exact cause of the absence of phase 2 flagellar antigen expression in *Salmonella* 4,[5],12:i:- is not known and likely varies by the origin and evolution of each isolate. The absence could be caused by a change in the promoter region of the *fljA* and *fljB* operons or in *fliA* or *hin* due to their roles in regulation of phase 2 flagellar expression[60,83,84]. As discussed previously with respect to the variation in the PCR testing results obtained by multiple studies of *Salmonella* 4,[5],12:i:- isolates, there is a significant amount of dissimilarity in the genetic

composition and gene expression of this serovar. The absence of phase 2 flagellar expression could originate from dysfunction in other parts of the process of flagellar expression aside from the sequence of the gene, including protein translocation, gene expression, and flagellar filament assembly[75].

The effect of the absence of the phase 2 flagellar expression on the ability of *Salmonella* 4,[5],12:i:- to cause clinical disease remains largely unknown. The outcome of infections with *Salmonella* Typhimurium in mice were much different, based on whether the bacteria were predominantly expressing *fljB* or *fliC* at the time the mice were inoculated[78]. Of the mice inoculated with primarily *fljB*-expressing *Salmonella* Typhimurium, 14 out of 14 mice survived to the completion of the study while only 10 out of 14 mice inoculated with primarily *fliC*-expressing *Salmonella* Typhimurium survived[78]. In addition, 87% of the *Salmonella* that was isolated from the *fliC*-expressing *Salmonella* Typhimurium inoculated mice were expressing *fliC*[78]. In contrast, only 60% of the *Salmonella* isolated from the Peyer's patches of 13 of the 14 mice infected with *fljB*-expressing *Salmonella* were still expressing *fljB*[78]. It is worth noting that in the spleen of mice that were infected with *fljB* expressing *Salmonella*, the majority of the mice were expressing primarily *fliC* by DPI 21[78]. It is possible that *fliC* provides a selective advantage in mice, although the translation to swine from this study is unclear.

Flagella, regardless of the phase, play an important role in the pathogenesis of *Salmonella*. Flagella are required for motility, which is subsequently required for a bacterium to interact with and invade a host cell[85]. There is also evidence that the flagella aids in selection of the target site of invasion, which would potentially alter the overall effect of that *Salmonella* infection on the host[85]. It seems logical to conclude that flagella are critical for the pathogenicity of *Salmonella* in the host, but the benefit or detriment of possessing two phases of flagella

remains unclear. To study this, an *in vitro* analysis was conducted comparing *Salmonella* 4,[5],12:i:- and *Salmonella* Typhimurium in porcine intestinal epithelial cells[86]. While the non-motile variant of *Salmonella* Typhimurium had a reduced ability to adhere to and invade the porcine intestinal epithelial cells when compared to biphasic *Salmonella* Typhimurium, the monophasic *Salmonella* 4,[5],12:i:- isolate evaluated in this study had equivalent adhesion and invasion capabilities[86]. However, there were biphasic *Salmonella* Typhimurium strains in this study that had an impaired adhesion and invasion ability[86], which indicates that there is also within-serovar variation that could not be thoroughly evaluated based on this data given the small number of isolates compared. It is also well known that flagella stimulate the release of signaling molecules to induce an inflammatory response in the intestine of the host[86-88]. TLR-5 and interleukin-8 were upregulated upon infection with *Salmonella* 4,[5],12:i:-, indicating that this serovar is equally antigenic to its biphasic counterpart[86,89]. In a recent study of eight pigs experimentally infected with a multidrug-resistant strain of *Salmonella* 4,[5],12:i:-, a disease similar to that commonly observed with *Salmonella* Typhimurium infection occurred, including fever and diarrhea[90].

Cause of emergence

One of the questions about *Salmonella* 4,[5],12:i:- that remains unanswered, and potentially can never be answered with complete certainty, is the underlying cause of its rapid increase in identification over the past couple of decades. Several theories have been proposed in the literature which will be discussed here. *Salmonella* 4,[5],12:i:- tends to have a high level of resistance to antimicrobials. Of 110 *Salmonella* 4,[5],12:i:- isolates from humans evaluated in the National Antimicrobial Resistance Monitoring System (NARMS) in 2014, 27.9%, 34.2%, 9.8%,

and 6.5% were resistant to three or more, four or more, five or more, and six or more antimicrobial classes per Clinical and Laboratory Standards Institute (CLSI), respectively[47]. The antimicrobial classes include aminoglycosides, beta-lactams, cephems, macrolides, penicillins, quinolones, folate pathway inhibitors, phenicols, and tetracyclines[47]. In 2014 human-origin *Salmonella* 4,[5],12:i:- isolates, greater than 50% of the isolates were resistant to one or more of the following antimicrobials: streptomycin, ampicillin, sulfisoxazole, and tetracycline[47]. When comparing this level of resistance to that of *Salmonella* Typhimurium, only 25% or less of the *Salmonella* Typhimurium isolates in the NARMS study were resistant to the same antimicrobials of concern as *Salmonella* 4,[5],12:i:-[47]. In a study of swine in Spain, 100% of the *Salmonella* 4,[5],12:i:- isolates from clinically ill pigs were considered multidrug resistant (resistant to four or more antimicrobials) while 84.6% of *Salmonella* 4,[5],12:i:- from clinically healthy pigs were multidrug resistant; both of these percentages were higher in *Salmonella* 4,[5],12:i:- than *Salmonella* Typhimurium in each of the populations (clinically healthy versus ill)[91]. There have been many other reports on the antimicrobial resistance of *Salmonella* 4,[5],12:i:- isolates from different species and different countries, all with the same conclusion that this serovar is generally highly resistant to antimicrobials[29,61,67,92]. Given the extensive use of antimicrobials in both humans and livestock production, it is possible that antimicrobial resistance may provide a selective advantage to the success of the monophasic serovar.

In addition to antimicrobial resistance, resistance to heavy metals may also have some role in the emergence of *Salmonella* 4,[5],12:i:-. Some strains of *Salmonella* 4,[5],12:i:- possess a genomic island known as SGI-3, which encodes genes that confer resistance to heavy metals such as copper sulfate and zinc[93]. Another study had similar findings in which the majority (44/50) of isolates were determined to be resistant to copper and silver due to the presence of the

pcoA-pcoD and *silA-silE* genes, respectively[94]. Some isolates have also been shown to be resistant to mercury and tolerant of cations and arsenic[94]. Given the increased use of heavy metals in livestock feed to replace the role of antimicrobials[53,54], the resistance to heavy metals may also provide some advantage to the serovars' survivability. Another cause may be that *Salmonella* 4,[5],12:i:- possesses a competitive advantage over *Salmonella* Typhimurium within and outside of the host. Outside of the host, the majority of the monophasic serovar isolates have been shown to possess the ability to form biofilms, which would enhance the survivability while reducing the effects of antimicrobials[95,96]. Although the biofilm formation ability may enhance the survival of *Salmonella* 4,[5],12:i:-, the role of this in the emergence of *Salmonella* 4,[5],12:i:- remains unclear as *Salmonella* Typhimurium has a similar biofilm formation ability[96]. *Salmonella* 4,[5],12:i:- also commonly possesses multiple virulence genes that could contribute to its survival within its host and environment; these genes include but are not limited to *sipC* which is involved in cell adhesion and invasion, *sopB* which is involved in the intestinal changes that lead to diarrhea, and *hilA* which activates the invasion process[97]. The combination of biofilm formation and presence of many virulence genes that function in the pathogenesis of *Salmonella* 4,[5],12:i:- may be the source of the competitive advantage *in vivo*. This is an area that needs further research to determine the exact cause for emergence, as this may help control this serovar in the animal reservoir and its transmission to humans.

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CHAPTER 2:

EMERGENCE OF *SALMONELLA* 4,[5],12:I:- AS THE PRIMARY SEROVAR IDENTIFIED FROM SWINE CLINICAL SAMPLES AND DEVELOPMENT OF A MULTIPLEX REAL-TIME PCR FOR IMPROVED *SALMONELLA* SEROVAR-LEVEL IDENTIFICATION

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Samantha A. Naberhaus[a,b,d,e], Adam C. Krull[b,d], Laura K. Bradner[b,d], Karen M. Harmon[b,d], Paulo Arruda[b,d], Bailey L. Arruda[b,d], Orhan Sahin[b,d], Eric R. Burrough[b,d], Kent J. Schwartz[b,d], Amanda J. Kreuder[b,c,d,e]

[a]Primary researcher and author

[b]Contributing author

[c]Author for correspondence

[d]Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA

[e]Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA

Abstract

The rapid identification of the infecting *Salmonella* serovar from porcine diagnostic samples is vital to allow implementation of appropriate on-farm treatment and management decisions. Although identification at the serogroup level can be rapidly achieved at most veterinary diagnostic laboratories, final *Salmonella* serovar identification often takes several weeks because of the limited number of reference laboratories performing the complex task of serotyping. *Salmonella* serogroup B, currently the dominant serogroup identified from swine clinical samples in the United States, contains serovars that vary from either highly pathogenic to minimally pathogenic in swine. We determined the frequency of detection of individual Group B serovars at the Iowa State Veterinary Diagnostic Laboratory (ISU-VDL) from 2008 – 2017, and validated a multiplex real-time PCR (rtPCR) to distinguish pathogenic serogroup B serovars from those of lesser pathogenicity. Our results indicate that, since 2014, *S.* 4,[5],12:i:- has been the dominant serovar identified from swine clinical samples at the ISU-VDL, with *S.* Typhimurium now the second most common serovar identified. A rtPCR was developed to allow rapid differentiation of samples containing *S.* 4,[5],12:i:- and *S.* Typhimurium from samples containing serovars believed to be of less pathogenicity, such as *S.* Agona and *S.* Derby. When combined with enrichment culture, this rtPCR has the ability to significantly improve the time to final serovar diagnosis of the 2 most commonly identified pathogenic *Salmonella* serovars in swine, and allows for rapid implementation of serovar-specific intervention strategies.

Introduction

Non-typhoidal *Salmonella enterica* infections in swine can result in clinical disease (i.e., salmonellosis) or asymptomatic infections, with clinical disease most commonly observed in weaned, growing, and finishing pigs.[23] Although clinical disease typically occurs as fever with diarrhea, the clinical manifestation of *Salmonella enterica* infection is highly dependent on the infecting serovar and age of the pigs.[19,23] Subclinical infections present unique diagnostic interpretation challenges given that pigs are infected with *Salmonella* without any clinical signs of disease but will often shed the organism in their feces for an extended period of time.[8,45] Variation between pigs and inconsistencies in shedding patterns make it difficult to confidently confirm that an animal is truly negative for a current *Salmonella* infection,[9,31] which poses a risk of continued environmental contamination leading to exposure of cohorts. Hence, it is not surprising that a high prevalence of non-typhoidal *Salmonella* serovars has been documented among finishing pigs at slaughter.[3,29,42]

Salmonella shedding around the time of slaughter, including transport and lairage, increases the risk of contamination of pork products with *Salmonella*. This is of concern given that *Salmonella enterica* is the most commonly reported cause of foodborne illness in humans.[11] There are significant differences in the contribution of each serovar to human disease risk; those that lack host restriction, such as *S.* Typhimurium, are of primary importance. *Salmonella* Typhimurium has been widely recognized as a cause of foodborne illness in humans for many years, especially with regard to contaminated pork products.[13,30,44] More recently, the monophasic variant of *S.* Typhimurium, *S.* 4,[5],12:i:-, has been implicated as an increasingly common cause of human illnesses.[6,24,33,36]

It is widely accepted that *S.* 4,[5],12:i:- is a variant of *S.* Typhimurium, based on molecular subtyping through pulsed-field gel electrophoresis, multilocus sequence typing, phage

typing, and plasmid characterization.[2,27,28,41] Although *S.* Typhimurium expresses the "i" phase 1 flagellar antigen and the "1 and 2" phase 2 flagellar antigens, the 4,[5],12:i:- serovar lacks phase 2 flagellar expression.[41] Although *S.* 4,[5],12:i:- is considered a *S.* Typhimurium variant, worldwide, it has been demonstrated that there are many clones originating from multiple independent events. These events resulted in deletions or mutations in various genomic regions including: *hin* (which encodes a recombinase that regulates inversion of the *fljAB promoter), fljB* (which encodes the phase 2 flagellar proteins), or the *fljAB* promoter region.[37,41] Minimal data exists at this time detailing the emergence of *S.* 4,[5],12:i:- in the United States. Of the 51 isolates recovered from swine between 2014-2016 that were evaluated via whole genome sequencing, 48 were part of a single emerging clade.[18] However, this work was limited to samples submitted from the midwestern United States only, and it was unclear how many production units were represented in the dataset and thus whether the study was representative of the current population of *S.* 4,[5],12:i:- circulating in the United States.

In a review of case data from clinical submissions to the ISU-VDL during an 18-mo period of 2016-2017, a statistically significant positive association between histologic lesions consistent with enteric salmonellosis and isolation of *Salmonella* 4,[5],12:i:- was noted.[1] A small-scale pathogenicity study utilizing a multi-drug resistant isolate from a human outbreak also reported the ability of *S.* 4,[5],12:i:- to cause disease in swine similar to that seen in previous studies utilizing *S.* Typhimurium in the same laboratory.[40] These findings are consistent with an *in vitro* study that compared the ability of an *S.* 4,[5],12:i:- isolate and an *S.* Typhimurium isolate to adhere to and invade cultures of porcine intestinal epithelial cells, determining that there was no difference between the 2 serovars in their cytotoxicity, colonizing ability, or effect on pro-inflammatory chemokine release.[14] Thus, without additional available data to the contrary, it

seems reasonable to consider *S.* 4,[5],12:i:- to be of similar pathogenicity to *S.* Typhimurium in swine. However, although vaccines to aid in the prevention of disease caused by *S.* Typhimurium in swine are available commercially in the United States, there are no vaccines similarly labeled for control of *S.* 4,[5],12:i:-. Given that no research has been published to date on the potential for cross-protection of vaccination against *S.* Typhimurium on the incidence of *S.* 4,[5],12:i:- in swine operations, and although pathogenicity may be equivalent, successful prevention strategies may not be identical between the serovars. In addition, evaluation of antimicrobial resistance patterns of *S.* 4,[5],12:i:- isolates from humans has shown these isolates to be more highly resistant to several different classes of antibiotics compared to human isolates of *S.* Typhimurium, thus effective treatment of clinical disease in swine may differ between the serovars.[11]

Both *S.* Typhimurium and *S.* 4,[5],12:i:- are part of *Salmonella* serogroup B. From the late-1990s through the mid-2000s, *S.* Typhimurium and *S.* Derby were reported as the most frequently isolated Group B serovars from swine in the United States.[20] Interestingly, *Salmonella* 4,[5],12:i:- was rarely identified prior to the mid-1990s.[41] This serovar first appeared in Europe, where it is now the third most frequently isolated serovar from human salmonellosis cases.[16] In the United States, it was the fifth most frequently isolated serovar from human enteric salmonellosis in 2014, increasing in prevalence by 194% from 2005 to 2015.[12] From July 2006 through June 2015, the Minnesota Veterinary Diagnostic Laboratory reported the most frequent *Salmonella* serovars isolated from swine sample submissions (n = 2,537) were *S.* Typhimurium var 5- (28.2%), *S.* Agona (14.7%), and *S.* Derby (12.1%).[26] The Minnesota study[26] as well as a national study incorporating both human and veterinary data[46] both noted an increase in isolation of *S.* 4,[5],12:i:- similar to that initially reported by the Iowa State University Veterinary

Diagnostic Laboratory (ISU-VDL) in late 2016 (Krull A, et al. Increased frequency of isolation of multi-drug resistant *Salmonella* I 4,[5],12:i:- from swine with histologic lesions consistent with salmonellosis. Proc Am Assoc Vet Lab Diagnosticians Ann Conf. Oct 2016. Greensboro, NC). According to the 2013 NARMS Retail Meat Interim Report, *S.* 4,[5],12:i:- was identified as one of the serovars most commonly isolated from retail pork in the United States.[12] It is worth noting that *S.* 4,[5],12:i:- has been detected in cattle[15,26,32] and poultry[12,15] in addition to swine.[5,26,43]

Rapid detection and identification of *Salmonella*, particularly serovars such as *S.* Typhimurium and *S.* 4,[5],12:i:- that are believed to be pathogenic to both pigs and humans, is critical for preventing foodborne illness outbreaks. Implementation of suitable treatment and prevention protocols on farms is reliant on rapid detection and identification of pathogens, which subsequently aids in reducing potential pork contamination. Current protocols for *Salmonella* isolation, identification, and serogrouping take 3-5 d on average.[34] However, serotyping at a reference laboratory can take 4-6 wk. The extended time to final identification at the serovar level limits the ability of veterinarians and producers to begin the most appropriate treatment and prevention protocols. Our objectives were: 1) to determine the frequency of detection of various Group B serovars commonly identified from swine at the ISU-VDL from 2008 to 2017, and 2) to develop a multiplex real-time PCR (rtPCR) to rapidly detect and differentiate *Salmonella* serovars likely to be pathogenic in swine (i.e., *S.* Typhimurium and *S.* 4,[5],12:i:-) from those of lesser pathogenicity (e.g., *S.* Derby) from clinical specimens.

Materials and methods

Retrospective analysis of ISU-VDL data

The ISU-VDL receives >75,000 case submissions annually, ~75% of which are samples from all types of swine production systems throughout the United States. The ISU-VDL Laboratory Information Management System provided data for the analysis of the frequency of detection of specific serovars of *Salmonella*, using search criteria which included: 1) the period from January 1, 2008 through December 31, 2017; 2) all porcine cases from which a *Salmonella* species was isolated from a clinical specimen (primarily feces or tissues); 3) the isolate was verified as *Salmonella* and the serovar identified by the National Veterinary Services Laboratories (NVSL); and 4) the isolate had antimicrobial susceptibility testing performed. All submissions considered research cases or cases in which *Salmonella* isolate serotyping was unable to be performed were eliminated from analysis. When multiple *Salmonella* isolates were isolated from a single case, the standard laboratory protocol was to send only a single isolate to NVSL for serotyping, therefore, each isolate listed in our analysis represents a unique case submission to the ISU-VDL.

Culture, isolation, and DNA extraction of bacterial samples

All bacterial isolates used for validation were selected from clinical samples submitted to the ISU-VDL. Porcine samples submitted for routine enteric culture were plated onto 4 different agar plates/atmospheric conditions for isolation of pathogens associated with enteric disease. These included 1) tryptic soy agar (TSA) with 5% sheep blood (Remel Products, Lenexa, KS) incubated under 5% CO_2, 2) TSA with 5% sheep blood incubated anaerobically, 3) brilliant green agar with novobiocin (BGN) (in-house) incubated aerobically, and 4) tergitol-7 agar (T7)

(Remel Products) incubated aerobically. All plates were incubated at 35°C for a minimum of 48 h. Colonies consistent with *Salmonella* were selected from the BGN and/or T7 plates, subcultured to TSA to obtain pure cultures, and saved in 10% glycerol stocks for long-term storage at -80°C for further use.

Upon request from a diagnostician with clinical suspicion of salmonellosis, a 24-h enrichment with either tetrathionate broth or buffered peptone water (BPW) was also included in select cases. For these samples, the enrichment culture was incubated at 42°C and then was sub-cultured onto BGN, which was incubated aerobically for 24 h.

Confirmation of suspect isolates was done via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) following the manufacturer's recommendations (Bruker Daltonics, Billerica, MA). A minimum MALDI-TOF-MS confidence score of 2.10 was required for confirmatory identification. Confirmation of *Salmonella* identification was then followed by serogrouping via slide agglutination testing (BD Diagnostics, Sparks, MD; SSI Diagnostica, Herredsvejen, Denmark) at ISU-VDL and serotyping at NVSL.

Bacterial isolates from routine cases were saved at -80°C in brain heart infusion (BHI) broth with 10% glycerin. Stored isolates selected for use in validation of the rtPCR were cultured onto TSA with 5% sheep blood agar and incubated at 35°C for 24 h to ensure purity prior to DNA extraction.

DNA extraction of pure culture of bacterial isolates was achieved first by suspension of multiple colonies of the culture in phosphate-buffered saline (PBS). DNA extraction of the samples was then performed (RNA DNA Pathogen extraction kit; Kingfisher rapid throughput DNA extraction system; Thermo Fisher Scientific, Rochester, MN) according to manufacturer's recommendations.

Development of rtPCR

The rtPCR assay was designed to detect the following genes or regions: *invA* (present in all *Salmonella*,[21] *fliA* (present in both *S.* Typhimurium and *S.* 4,[5],12:i:-),[17,25] *fljB* (present in *S.* Typhimurium, may be absent in *S.* 4,[5],12:i:-),[7,29,28,41] the intergenic space between *hin* and *iroB* (present in *S.* Typhimurium, may be absent in *S.* 4,[5],12:i:-),[7,29,41] and an internal control (see Table 1). To be considered *S.* 4,[5],12:i:-, one or both of either the *fljB* or *hin-iroB* targets must be absent (negative) to result in the monophasic variant. The primers and probes were designed and validated previously (Supplementary Table 1).[35,38] Controls for each rtPCR assay included an internal amplification control, 2 positive extraction controls, a negative extraction control, and a negative amplification control. The internal amplification control consisted of G-block gene fragments 125-500 bp (XIPC_IVT; Integrated DNA Technologies; 149551620) based on the GenBank sequence DQ883679, as described previously.[39] The positive extraction controls included one *S.* Typhimurium isolate and one *S.* 4,[5],12:i:- isolate, both at a concentration of 10^4 CFU/mL suspended in Luria-Bertani (LB) broth. The negative extraction control was nuclease-free water (Life Technologies, Waltham, MA). The negative amplification control was the prepared rtPCR master mix.

Real-time PCR assays were carried out in 25 µL reactions (QuantiTect Virus + ROX kit; Qiagen, Waltham, MA) according to the manufacturer's recommendations, with each target primer at a final concentration of 200 nM, each target probe at 100 µM, and XIPC primers and probe at final concentrations of 160 and 60 nM, respectively. A Rotor Gene-Q 5-plex HRM thermocycler system (Qiagen) was used with the following cycling conditions: 20 min at 50°C, 5 min at 95°C, then 40 cycles of alternating 15 s at 95°C and 1 min at 60°C. The initial 20-min step was included to allow simultaneous testing with other reverse-transcription rtPCRs run in

the testing laboratory. The threshold for analysis was set at 0.02. The following interpretation criteria were used for all sample types. The negative cut-off for detection of any *Salmonella* DNA, regardless of serovar, present in the sample was maintained at a cycle threshold (Ct) value of 40, as is standard in the ISU-VDL; the sample was considered positive for the presence of any *Salmonella* DNA below this threshold. A sample was considered positive and able to be identified at the serovar level if the Ct was <30. If the Ct was 30-40, it was considered positive for *Salmonella* DNA but inconclusive for serovar identification. Therefore, further testing utilizing additional culture and/or enrichment followed by NVSL serotyping or repetition of rtPCR on pure culture was necessary for serovar identification in some samples.

Inclusivity and exclusivity

To assess the inclusivity of the rtPCR, 61 *S.* 4,[5],12:i:- isolates and 45 *S.* Typhimurium isolates were tested. To assess the exclusivity of the rtPCR, 38 isolates, representing 28 non-Typhimurium, non-4,[5],12:i:- serovars of *Salmonella* and 7 non-*Salmonella* organisms commonly found in feces, were tested (Supplementary Table 2). *Salmonella* serovars selected for exclusivity testing included the 10 serovars isolated most commonly from porcine samples at the ISU-VDL as well as serovars that are closely related to the serovar Typhimurium. All isolates tested were obtained from previous ISU-VDL case submissions using the culture techniques described above.

Limit of detection studies

Two limit of detection studies were completed, one with and one without enrichment in BPW prior to DNA extraction and rtPCR. Enrichment in BPW has been shown in the ISU-VDL

to be a superior method, relative to tetrathionate broth, for enriching for *Salmonella* sp. in clinical and environmental samples prior to rtPCR (Krull A, et al. Use of enrichment and quantitative PCR to improve detection of *Salmonella* in referral hospitals. Am Coll Vet Intern Med (ACVIM) Forum Research Report, June 2016, Denver, CO). To determine the non-enriched limit of detection, the following method was repeated with one *S.* Typhimurium and one *S.* 4,[5],12:i:- isolate each using *Salmonella*-negative feces (confirmed by enrichment culture) as the sample medium. To prepare the dilutions, a 0.5 McFarland standard (~1.5 x 10^8 CFU/mL) was prepared from a pure culture and then serially diluted 1:10 in PBS. The actual amount of *Salmonella* in each dilution was determined using the standard plate count method on TSA with 5% sheep blood agar. *Salmonella*, in the form of 0.5 mL of the serially diluted McFarland standard, was added to 0.2 gram of porcine feces to create 10-fold dilutions of *Salmonella* from 5 x 10^7 to 5 x 10^1 CFU/mL. Each dilution was created in triplicate. *Salmonella* was also added to feces to create 5-fold dilutions of *Salmonella* from $1x10^4$ – $1x10^1$ CFU/mL, again in triplicate. DNA from the fecal samples was extracted using the bead beating method recommended in the ThermoFisher Total Nucleic Acid kit insert, using the RNA DNA Pathogen Extraction kit and 100 µm acid-washed zirconium bead-filled tubes (OPS Diagnostics, Lebanon, NJ).

The post-enrichment limit of detection was determined by adding 250 µL of the fecal samples created for the non-enrichment limit of detection studies to 5 mL of BPW. The inoculated BPW was then incubated at 35°C for 18-24 h prior to DNA extraction and rtPCR. Post-enrichment DNA samples in BPW were extracted using the DNA extraction method described above for pure culture.

Sequencing of isolates with unexpected results

Salmonella isolates that did not react as expected on the rtPCR (i.e., results did not match NVSL serotyping) were sequenced in the region amplified by the *fljB* primer set to determine the cause of the unexpected result. Sanger sequencing was completed at the ISU DNA Facility. The sequences were then assembled for further analysis using DNASTAR. BLAST analysis was subsequently used to compare the consensus sequences to known DNA sequences of *S.* Typhimurium and *S.* 4,[5],12:i:-.

Validation using clinical samples (retrospective and prospective)

Further validation of the rtPCR was done via retrospective and prospective analysis of clinical samples submitted to the ISU-VDL. For retrospective validation, porcine cases that met the following criteria were selected: 1) *Salmonella* was isolated from the sample submitted for culture, and 2) molecular testing was performed for identification of other disease entities on a sample from the same pig that had a *Salmonella*-positive culture. The use of samples that had molecular testing performed ensured that there was a DNA extract stored at -20°C from which further testing could be completed. The previously extracted DNA was tested via the *Salmonella* rtPCR as described above to compare to the results of culture.

For prospective validation, feces, colonic mucosal scrapings, and fecal swabs rinsed in 1 mL PBS were collected from pigs that had gross lesions suggestive of salmonellosis upon postmortem examination. These samples were subsequently tested for presence of *Salmonella* both directly and post BPW enrichment using the rtPCR. Other tissues from the large intestine were also collected for testing that included standard culture for *Salmonella*. Cultured isolates

from the standard testing were confirmed as *Salmonella* by the ISU-VDL and serotyped by the NVSL.

Results

Retrospective data analysis

A total of 10,194 isolates of *Salmonella* were confirmed from swine clinical cases at the ISU-VDL during 2008-2017. Of these, 3,476 of 10,194 (34%) of the isolates did not include the state of origin of the sample on the submission form. The remaining 6,718 of 10,194 (66%) of *Salmonella* isolates originated from farms in the following states: Iowa (45%); North Carolina (17%); Illinois (6%); Minnesota (5%); Arkansas, Kansas, Missouri, and Virginia (4% each); Nebraska and Pennsylvania (2% each); Arizona, California, Colorado, Hawaii, Idaho, Kentucky, Maryland, Massachusetts, Michigan, Montana, New Hampshire, New York, North Dakota, Ohio, Oklahoma, South Carolina, South Dakota, Texas, Utah, Wisconsin, and Wyoming (0.01-1% each).

From 2008 to 2017, the number of *S.* 4,[5],12:i:- isolates identified from clinical cases in swine by the ISU-VDL rapidly increased, from a total of only 26 isolates between January 1, 2008 through December 31, 2010, to 331 isolates identified in 2017 alone. During the same timeframe, isolation of *S.* Typhimurium decreased from 364 isolates in 2008 to 144 isolates in 2017. In 2008, from a total of 1,060 isolates, the 5 *Salmonella* serovars most commonly isolated from swine through the ISU-VDL were (in order): Typhimurium (34%, serogroup B); Derby (13%, serogroup B); Choleraesuis (9%, serogroup C1); Agona (7%, serogroup B); and Heidelberg (6%, serogroup B). During 2008, *Salmonella* 4,[5],12:i:- was the 5th most commonly identified serogroup B isolate and the 13th most commonly isolated serovar overall, representing

<2% of all isolates of *Salmonella* from swine. In contrast, by 2017, the 5 most commonly isolated serovars, from among 1,031 total isolates, were (in order): 4,[5],12:i:- (32%); Typhimurium (14%); Derby (9%); Choleraesuis (7%); and Infantis (5%; serogroup C1). Thus, by 2017, 32.1% of all *Salmonella* isolated from swine at the ISU-VDL were identified as *Salmonella* 4,[5],12:i:- . Additionally, *S.* 4,[5],12:i:- made up 50.3% of all Group B isolates (Fig. 1). This observation coincided with a proportional decrease in identification of both *S.* Typhimurium (decreased from 34.3% in 2008 to only 14.0% of isolates in 2017) and all other *Salmonella* serogroup B serovars as well. Interestingly, the percentage of serogroup B isolates from porcine samples has remained relatively constant between 2008 and 2017, comprising 65% of 1,060 total porcine *Salmonella* isolates in 2008 and 64% of 1,031 total isolates in 2017. The tipping point in observed dominance between *S.* Typhimurium and *S.* 4,[5],12:i:- in swine occurred rapidly between 2013 and 2014, and the trend for increasing actual and relative frequency of isolation of *Salmonella* 4,[5],12:i from swine cases has remained constant since that time.

Inclusivity and exclusivity of the rtPCR

Results of the inclusivity study indicated that, of the 45 serovar-confirmed *S.* Typhimurium isolates tested, 44 were correctly identified as *S.* Typhimurium by the rtPCR assay based on a positive signal for all 4 genes tested (Supplementary Table 3). Of the 61 *S.* 4,[5],12:i:- isolates tested, 60 were correctly identified as matching the NVSL-confirmed serovar (4,[5],12:i:-) based on a positive signal for the *invA* and *fliA* genes and negative on either one or both of the *hin-iroB* or *fljB* targets given that one or both of which must be absent for the isolate to be considered monophasic. Isolates tested for exclusivity reacted as expected (Supplementary Table 2), with all

Salmonella isolates testing positive for the *invA* gene, negative for *fliA* gene, and variable results for the other 2 targets. All non-*Salmonella* fecal organisms tested for exclusivity also reacted as expected, being negative for all gene targets.

Interestingly, the *S.* 4,[5],12:i:- isolates were consistently a minimum of 8 Ct values higher for the *fljB* target than the Ct value of the *invA* and *fliA* gene targets (Table 2). This trend was not noticed in the *S.* Typhimurium isolates. To determine the source of the differences in the Ct values of *fljB*, 5 *S.* Typhimurium and 5 *S.* 4,[5],12:i:- isolates were sequenced in the region amplified by the *fljB* primers which includes the 5' coding region of the *fljB* gene. The *fljB* gene is 1,521 nucleotides, translating to 506 amino acids. Four base pairs were found to be consistently different between *S.* Typhimurium and *S.* 4,[5],12:i:- isolates (nucleotide 38: C to T; nucleotide 73: A to T; nucleotide 103: T to C; and nucleotide 163: C to T). The base pair differences did not result in any changes in the translated amino acid sequence. Additionally, the base pair differences were not located in the primer or probe binding sites, so it is unclear why the change results in differences in Ct values in the rtPCR assay. Several other *Salmonella* serovars tested on exclusivity testing also exhibited the same difference in Ct values between targets (Supplementary Table 2).

For the 2 isolates tested for inclusivity that did not react as expected in the rtPCR, further sequencing was also performed (Supplementary Table 3). Isolate "A" was positive for all 4 targets, but the *fljB* gene Ct value was >8 cycles greater than the *invA* and *fliA* genes. The Ct value difference would make the isolate appear to be a *S.* 4,[5],12:i:- isolate, which matches its identification by NVSL serotyping. However, given that the isolate was positive for all 4 genes by the rtPCR, identification as a *S.* Typhimurium isolate would also be expected. Isolate "B" was negative for *hin-iroB* but positive for the remaining 3 targets. Given that the isolate was negative

for *hin-iroB*, it would be expected to be a *S.* 4,[5],12:i:- isolate. However, the lack of a Ct difference between the *fljB* and *invA/fliA* genes would make the isolate more likely a *S.* Typhimurium, which matches its identification by NVSL serotyping. When the sequence of the target region amplified by the *fljB* primers was determined for both isolates, the identification based on the 4 base pair differences matched the NVSL serovar identification.

Limit of detection and clinical validation of rtPCR

The limit of detection was determined to be ~ 500 CFU/g (or mL) of feces tested directly without enrichment. When the same samples were enriched in BPW for 18-24 h prior to running the rtPCR, the limit of detection was 5 CFU/mL feces. The calculated R^2 value for *S.* Typhimurium was 0.967 and for *S.* 4,[5],12:i:- was 0.964, indicating a strong inverse correlation between Ct values and CFU/mL (or g) of sample (Supplementary Fig. 1).

Further clinical validation of the rtPCR was completed using both retrospective (no enrichment) and prospective (both direct and enrichment) samples from clinical cases at the ISU-VDL (Table 3). All prospective samples (n = 24) were identified as the same by culture and rtPCR (both with and without enrichment). Specifically, 4 samples were identified as *S.* 4,[5],12:i:- in both culture and rtPCR. Four samples were identified as *Salmonella* Heidelberg in culture and as non-Typhimurium, non-4,[5],12:i:- *Salmonella* in rtPCR. Sixteen of the samples were negative for *Salmonella* in culture and rtPCR. Although there were differences in the Ct values obtained from the non-enriched version and enriched version of each sample, the same conclusion was reached on all of the samples (data not shown).

For the retrospective validation completed on previously extracted DNA, of the 42 samples tested, 34 samples gave identical matches between the culture and rtPCR results. Four of

the samples contained very low levels of *Salmonella* organisms in culture with only a few *Salmonella*-suspect colonies noted on the original agar plates; all of these samples were negative for *Salmonella* by rtPCR. Of the 4 remaining samples, 2 had high Ct values on rtPCR (30-40) that would have led to an interpretation as positive for the presence of *Salmonella* DNA in the sample but inconclusive for identification at the serovar level; these samples were positive for *S.* 4,[5],12:i:- by culture with only single-to-low colony growth present. The remaining 2 samples were classified as *S.* Typhimurium by rtPCR but as *S.* 4,[5],12:i:- by culture and serotyping.

Discussion

Our investigation of the most common serovars identified at the ISU-VDL clearly demonstrates that over the last several years, *S.* 4,[5],12:i:- has become the dominant *Salmonella* serovar isolated from clinical samples in swine at the ISU-VDL. This finding may have important implications for development of herd infection prevention strategies given that there are currently no vaccines labeled for control of infection with *Salmonella* 4,[5],12:i:- in swine in the United States. Additional research investigating the cause of the emergence of *S.* 4,[5],12:i:- is warranted based on our results.

The *fljB* gene did not react as was expected based on the results from the original published validation.[35] During our validation of the rtPCR, this gene was consistently 8-10 Ct cycles greater than that of the *fliA* and *invA* genes for all *S.* 4,[5],12:i:- isolates. Through sequencing of 5 *S.* Typhimurium and 5 *S.* 4,[5],12:i:- isolates, we noted that 4 base pairs were consistently different between the 2 serovars. However, none of the differences were in the primer or probe binding regions, and therefore, they do not help to explain this anomaly. It remains unknown why the difference was not observed in the original study.[35] However, it is

possible that, because the isolates originated from Germany for the original validation, they possess different mutations or deletions that led to the monophasic phenotype compared to the isolates from the United States that we used. The isolates used in the prior study also originated from human, animal, food, and environmental sources rather than strictly from porcine samples as was the case in our study. It is also possible that in the original validation, samples were only weakly positive for *Salmonella*, resulting in Ct values for the *fljB* gene greater than the negative cutoff. However, based on the consistency of the results achieved in our study, the Ct value difference can be used as an identification aid in differentiating *S.* Typhimurium isolates from *S.* 4,[5],12:i:- isolates. Although we do not believe that that this is a significant limitation to the assay based on the current knowledge of mutations that have led to the monophasic phenotype, it is possible that additional mutations exist and have yet to be characterized or will emerge that might interfere with identification utilizing our method. In addition, as all clones of *S.* 4,[5],12:i:- are at this time believed to originate from *S.* Typhimurium, at minimum all should still be identified as potentially pathogenic based on positivity of the *invA* and *fliA* genes via the rtPCR.

Due to variability between Ct values of the gene targets, one of the limitations of our rtPCR assay is that samples could not be identified reliably at the serovar level by our rtPCR if the Ct values were >30. Slight differences in Ct cycles between *invA*, *fliA*, and *hin-iroB*, as well as *fljB* in non-*S.* 4,[5],12:i:- isolates, raises concerns that as the standard Ct cutoff value (40) for negative rtPCR tests is approached, it is possible that one of the targets will appear negative while others test positive. However, we mitigated this issue by using a BPW enrichment step prior to the rtPCR to increase the amount of *Salmonella* DNA present in the sample, thereby avoiding high Ct values and ensuring more accurate interpretation of results. Based on this limitation, although the rtPCR can be used directly on clinical samples such as feces, intestinal

contents, and intestinal scrapings, the more ideal approach is to utilize the rtPCR either following BPW enrichment or as a confirmatory step following standard culture. In our study, use of the BPW enrichment step prior to rtPCR appears to provide advantages for both increasing sensitivity by improving the limit of detection (from 500 CFU/mL to 5 CFU/mL) and improving specificity by ensuring that enough organism is present to generate Ct values less than <30. The lack of enrichment prior to DNA extraction in the retrospective case study highlights the sensitivity challenges with rtPCR when a low number of *Salmonella* are present in a sample.

One additional potential limitation of our rtPCR applies to simultaneous infections by more than one serovar of *Salmonella*, which has been described in pigs and may in fact be commonplace.[22] The identification of *S.* 4,[5],12:i:- by rtPCR is dependent on the *fljB* and/or *hin-iroB* targets being negative. Therefore, if a pig had a co-infection with both *S.* 4,[5],12:i:- and a serovar that possessed those genes, the rtPCR results would be indistinguishable from a pig infected only with *S.* Typhimurium. We believe that the possibility of mixed infections may explain the difference between rtPCR identification and serotyping observed in 2 of the samples in the retrospective validation that was performed. The standard *Salmonella* culture protocol of the ISU-VDL includes pursuit of complete identification at the serovar level for only one isolate per sample. Thus, in the case of mixed infection, it is conceivable that the one serotyped isolate was not representative of the entire *Salmonella* population present in the original sample. While we do not at this time know the significance of mixed infections with *S.* Typhimurium and *S.* 4,[5],12:i:- in swine, discordant results between NVSL serotyping and rtPCR results in clinical cases may warrant further investigation to determine if multiple serovars may be present in a clinical sample to better direct clinical decision making. Additional research studies have also demonstrated that some isolates of *Salmonella* may present phenotypically as monophasic

isolates, but still maintain a biphasic molecular status.[4,10] Therefore, it is also possible that the few discordant results identified in our study may also be due to this phenomenon; further research into the reason for this is warranted.

Although our rtPCR cannot replace antimicrobial susceptibility testing in selection of the proper antimicrobial agent, it can detect the presence of *Salmonella* in a sample and identify it to the serovar level for *S.* Typhimurium and *S.* 4,[5],12:i:-. This identification can be completed earlier than susceptibility data can be made available. Given that *S.* 4,[5],12:i:- tends to be a highly resistant organism when compared to *S.* Typhimurium,[11] serovar identification provides additional information regarding the common antimicrobial resistance profile to aid in the earlier selection of an antimicrobial likely to be effective, which can minimize the overall effects of an outbreak in a herd. Recognized differences in common antimicrobial susceptibility patterns between the serovars may also provide cause for treatment failures in mixed infections.

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Tables

Table 1. Expected multiplex real-time PCR results for *Salmonella enterica* serovars Typhimurium and 4,[5],12,i:- compared to all other serovars based on presence or absence of target genes.

Gene target	Interpretation	Expected PCR Result		
		***Salmonella* 4,[5],12,i:-**	***Salmonella* Typhimurium**	***Salmonella* non-Typhimurium, non-4,[5],12:i:-**
invA	Present in all *Salmonella*	Positive	Positive	Positive
fliA	Present in both Typhimurium and 4,[5],12:i:- only	Positive	Positive	Negative
hin-iroB	Always present in Typhimurium, may be absent in 4,[5],12:i:- and other serotypes	*Positive or negative	Positive	Positive or negative
fljB†	Always present in Typhimurium, may be absent in 4,[5],12:i:- and other serotypes	*Positive or negative	Positive	Positive or negative

* *S.* 4,[5],12:i:- can be negative for either *hin-iroB*, *fljB*, or both *hin-iroB* and *fljB*

† If *fljB* has >8 threshold cycle (Ct) difference from *fliA* gene, then the isolate is likely *S.* 4,[5],12:i:-; if there is <5 Ct difference, then the isolate is likely *S.* Typhimurium

Table 2. Primer and probe sequences utilized for the multiplex real-time PCR for differentiation of *Salmonella* Typhimurium and 4,[5],12:i:- serovars.

Component	Gene and sequence	PCR product (bp)	Reference
	invA		
Forward Primer	CAT TTC TAT GTT CGT CAT TCC ATT ACC	133	Pusterla et al.[38]
Reverse Primer	AGG AAA CGT TGA AAA ACT GAG GAT TCT		
Probe	56-FAM-TCT GGT TGA-ZEN-TTT CCT GAT CGC ACT GAA TAT C-3IABkFQ		
	fliA		
Forward Primer	CAT TAC ACC TTC AGC GGT AT	254	Maurischat et al.[35]
Reverse Primer	CTG GTA AGA GAG CCT TAT AGG		
Probe	5Cy55-CGG CAT GAT TAT CCG TTT CTA CAG GG-3IAbRQSp		
	hin-iroB		
Forward Primer	GTG TGG CAT AAA TAA ACC GA	274	Maurischat et al.[35]
Reverse Primer	AGG CTT ACC TGT GTC ATC CA		
Probe	5Hex-TAA CGC GCT-ZEN-CAC GAT AAG GC-3IABkFQ		
	fljB		
Forward Primer	TGG TGC TGT TAG CAG AC	297	Maurischat et al.[35]
Reverse Primer	TCA ACA CTA ACA GTC TGT CG		
Probe	5TexRd-XN-AAC CGC CAG TTC ACG CAC-3IAbRQSp		
	IAC (XIPC695)		
Forward Primer	TTC GGC GTG TTA TGC TAA CTT C	NA	ISU-VDL internal control
Reverse Primer	GGG CTC CCG CTT GAC AAT A		

Probe	5Cy5-CTC CGC AGA-TAO-AAT CCA GGG TCA CG-3IAbRQSp		

IAC = internal amplification control; bp = base pairs

Table 3. Results of real time PCR exclusivity testing to ensure no cross reactivity of non-Typhimurium, non-4,[5],12:i:- serovars of *Salmonella* or other non-*Salmonella* organisms commonly found in feces.

Serotype	n	*invA*	*fliA*	*hin-irob*	*fljB*
S. Agona	2	+	-	-	-
S. Derby	2	+	-	-	-
S. Heidelberg	2	+	-	+	+
S. Infantis	2	+	-	+	+
S. Johannesberg	2	+	-	-	+
S. Anatum*	2	+	-	+	+
S. Seftenberg	2	+	-	-	-
S. Worthington*	2	+	-	+	+
S. Ohio*	2	+	-	+	+
S. Mbandaka*	2	+	-	+	+
S. Muenchen*	1	+	-	+	+
S. Brandenburg	1	+	-	-	+
S. Give	1	+	-	-	+
S. Havana	1	+	-	-	-
S. 6,7:Nonmotile	1	+	-	+	+
S. Saintpaul	1	+	-	+	+
S. Kiambu	1	+	-	+	+
S. Bovismorbidificans	1	+	-	-	+
S. Krefeld*	1	+	-	+	+
S. Litchfield	1	+	-	+	+
S. Liverpool	1	+	-	+	+
S. Cerro	1	+	-	-	-
S. London	1	+	-	+	+
S. Schwarzengrund	1	+	-	-	+
*S. R*isen	1	+	-	-	-
S. Newport*	1	+	-	+	+
S. Choleraesuis var kunzendorf	1	+	-	+	+
S. Uganda*	1	+	-	+	+
E. coli	1	-	-	-	-
Citrobacter freundii	1	-	-	-	-
Klebsiella pneumoniae	1	-	-	-	-
Enterobacter sp.	1	-	-	-	-
Enterococcus faecium	1	-	-	-	-
Streptococcus suis	1	-	-	-	-
Proteus mirabilis	1	-	-	-	-

* Ct value was >8 more than Ct value of *fliA* and *invA*, similar to the findings with *S.* 4,[5]12:i:-

Table 4. Results of real time PCR inclusivity testing of *Salmonella* Typhimurium and 4,[5],12:i:- isolates to ensure correct identification of serovar compared to traditional serotyping.

Serovar	n	*invA*	*fliA*	*hin-iroB*	*fljB*
Salmonella 4,[5],12:i:-	61	+	+	-	+*
Salmonella. Typhimurium	45	+	+	+	+
Isolate A (*S.* 4,[5],12:i:-)	1	+	+	+	+*
Isolate B (*S.* Typhimurium)	1	+	+	-	+

*Denotes that cycle threshold (Ct) value was >8 more than Ct value of *fliA* and *invA*

Table 5. Representative example of real time PCR results, expressed as cycle threshold (Ct) values, for pure culture of 4 different *Salmonella* serovars including *S.* Typhimurium and *S.* 4,[5],12:i:-.

Serovar	*Representative example of Ct values for each gene target*			
	invA	*fliA*	*hin-iroB*	*fljB*
S. 4,[5],12:i:-	22.98	24.62	Negative	35.2
S. Typhimurium	21.27	21.32	23.18	21.9
S. Agona	18.55	Negative	Negative	Negative
S. London	18.32	Negative	18.4	20.9

Table 6. Results of prospective and retrospective validation of the multiplex real time PCR completed directly on clinical samples and compared to NVSL serotyping results.

n	Identification	
	Culture result	rtPCR result
	Prospective Validation	
4	*S.* 4,[5],12:i:-	*S.* 4,[5],12:i:-
4	*S.* Heidelberg	*Salmonella*, non-Typhimurium, non-4,[5],12:i:-
16	No *Salmonella*	No *Salmonella*
	Retrospective Validation	
16	*S.* 4,[5],12:i:-	*S.* 4,[5],12:i:-
5	*S.* Typhimurium	*S.* Typhimurium
13	*Salmonella*, non-Typhimurium, non-4,[5],12:i:-	*Salmonella*, non-Typhimurium, non-4,[5],12:i:-
3	*S.* 4,[5],12:i:-*	Negative
1	*Salmonella*, non-Typhimurium, non-4,[5],12:i:-*	Negative
2	*S.* 4,[5],12:i:-*	Inconclusive†
2	*S.* 4,[5],12:i:-	*S.* Typhimurium

* Few colonies to low levels of *Salmonella* spp. growth in culture

†Results would have been interpreted as "Inconclusive" due to high cycle threshold (Ct) values >30

Figures

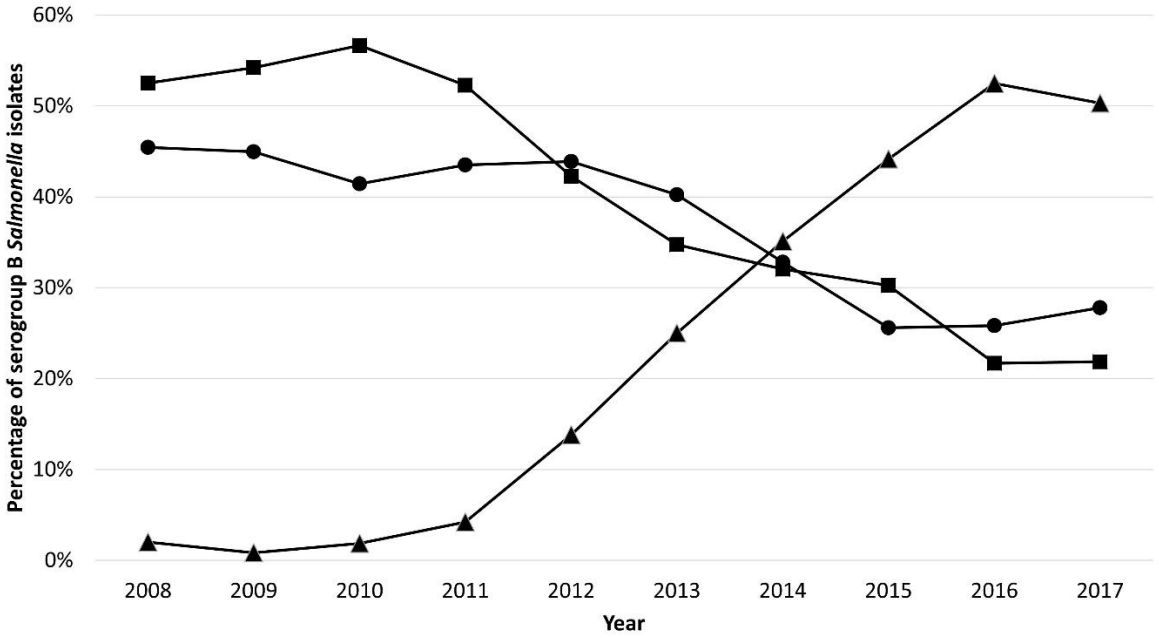

Figure 1. The percent contribution of *Salmonella* Typhimurium and 4,[5],12:i:- to the serogroup B *Salmonella* isolates of *Salmonella* identified from swine clinical cases at the Iowa State Veterinary Diagnostic Laboratory from 2008-2017.

Legend

Serogroup B *Salmonella*, non-Typhimurium, non-4,[5],12:i:-

Salmonella 4,[5],12:i:-

Salmonella Typhimurium

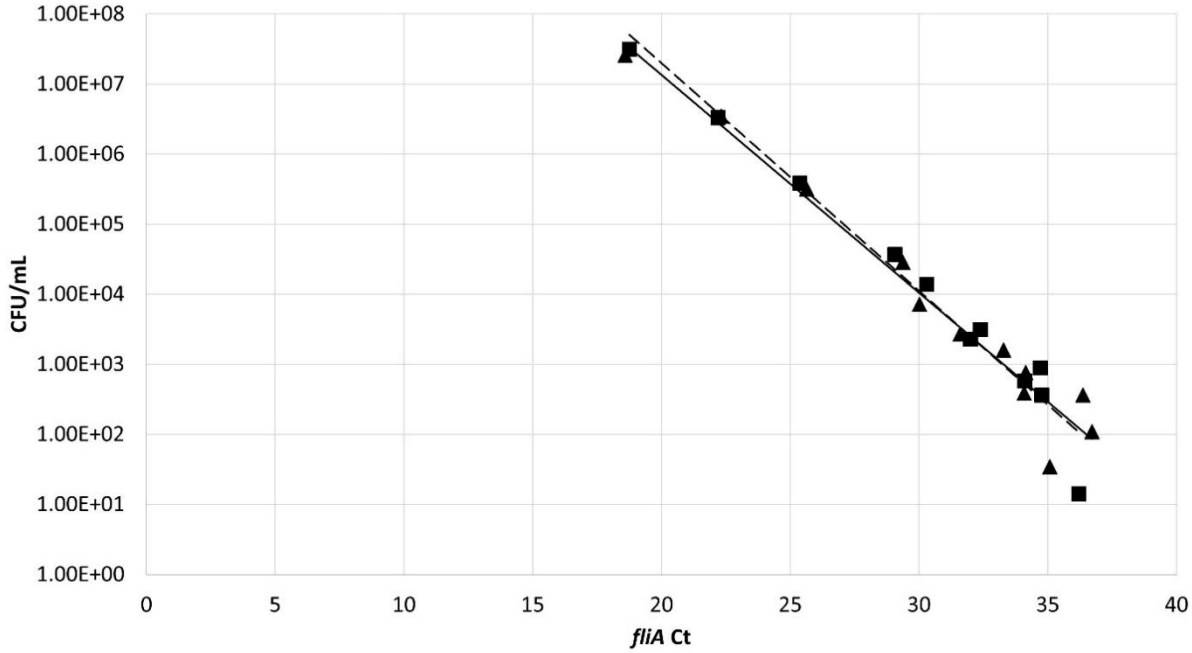

Figure 2. Determination of the limit of detection of the *fliA* probe of the multiplex real time PCR assay compared to standard plate counts to identify *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:- following inoculation into *Salmonella*-negative feces.

Legend

▲ *Salmonella* 4,[5],12:i:-

■ *Salmonella* Typhimurium

——— *Salmonella* 4,[5],12:i:- trend line

– – · *Salmonella* Typhimurium trend line

CHAPTER 3:

INVESTIGATION OF PATHOGENICITY AND COMPETITIVE FITNESS OF *SALMONELLA ENTERICA* SEROVAR 4,[5],12:I:- COMPARED TO *SALMONELLA* TYPHIMURIUM AND *SALMONELLA* DERBY IN SWINE

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Samantha A. Naberhaus[a,b,d,e], Adam C. Krull[b,d,], Bailey L. Arruda[b,d,], Paulo Arruda[b,d], Orhan Sahin[b,d], Kent J. Schwartz[b,d], Eric R. Burrough[b,d], Drew R. Magstadt[b,d], Franco Matias Ferreyra[b,d], Igor H. Gatto[b,d], Henrique Meiroz de Souza Almeida[b,d], Chong Wang[b,d,f], Amanda J. Kreuder[b,c,d,e]

[a]Primary researcher and author

[b]Contributing author

[c]Author for correspondence

[d]Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA

[e]Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA

[f]Department of Statistics, College of Liberal Arts and Sciences, Iowa State University, Ames, Iowa, USA

Abstract

Over the past 5 years, identification of *Salmonella* 4,[5],12:i:- in swine samples submitted to veterinary diagnostic laboratories in the United States has increased substantially while identification of *Salmonella* Typhimurium has concurrently decreased. The overall goal of this study was to facilitate a better understanding of the significance of increasing identification of this emerging serovar. To compare the pathogenicity of *S.* 4,[5],12:i:- in swine to the known pathogenic *S.* Typhimurium and lesser pathogenic *S.* Derby, 72 pigs (20 per *Salmonella* serovar treatment and 12 controls) were inoculated with either *Salmonella* Typhimurium, *Salmonella* 4,[5],12:i:-, *Salmonella* Derby or sham-inoculated and followed for up to 28 days after inoculation. To compare the competitive fitness of *S.* 4,[5],12:i:- to *S.* Typhimurium in swine when co-infected, a second animal study utilizing 12 pigs co-inoculated with equal concentrations of both *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i was performed.. The results clearly demonstrate that *Salmonella* serovar 4,[5],12:i:- possesses similar ability as *Salmonella* Typhimurium to cause significant clinical disease in swine and can be carried in the tonsils and ileocecal lymph nodes and shed in the feces of infected animals for weeks following exposure and illness. Additionally, when co-inoculated, 4,[5],12:i:- was consistently detected in the feces of a higher percentage of pigs and at higher levels than Typhimurium. This finding indicates that serovar 4,[5],12:i:- may have an improved competitive fitness relative to serovar Typhimurium when inoculated simultaneously into naïve pigs, which may suggest a mechanism for the increasing identification of *Salmonella* 4,[5],12:i:- in swine diagnostic samples over the past several years worldwide.

Introduction

Salmonella infections in swine, known as salmonellosis, can cause septicemia, enterocolitis, or subclinical infections[1,2]. The septicemic form is often caused by *S.* Choleraesuis and generally has high mortality, low morbidity, and signs including anorexia, fever, lethargy, and dyspnea[1]. The enterocolitic form has historically been associated with *S.* Typhimurium and generally has low mortality, high morbidity, and signs including anorexia, fever, lethargy, and diarrhea[1]. The subclinical form of salmonellosis does not cause overt signs of disease but may be associated with reduced productivity and average daily gain[3] in addition to increasing the risk of contamination of the final product during harvest thereby presenting a food safety concern.

With the characteristics of infection and outcome determined partly by the infecting serovar, a more thorough understanding of the pathogenesis of disease caused by highly prevalent serovars can aid in understanding the expected course of disease and appropriate control measures. In previous years, *S.* Typhimurium was the most commonly reported serovar in humans and swine[4,5]. More recently, however, *S.* 4,[5],12:i:- has been increasingly identified worldwide in humans, swine, cattle, and poultry[5-7]. In fact, *S.* 4,[5],12:i:- has been documented to be more common than *S.* Typhimurium in the US swine population based on data from the National Veterinary Services Laboratory (NVSL) and the Iowa State University (ISU) Veterinary Diagnostic Laboratory (VDL) since 2014[6] (see also CHAPTER 2). Although *S.* 4,[5],12:i:- has been reported more frequently, additional research needs done to evaluate the specifics of its disease-causing ability in swine. Based on published research using multiple-locus variable number tandem repeat analysis (MLVA), phage typing, pulsed-field gel electrophoresis (PFGE), and polymerase chain reaction (PCR), it is highly likely that *S.* 4,[5],12:i:- is a monophasic variant of *S.* Typhimurium[8-15]. Thus, *S.* 4,[5],12:i:- could resemble

the disease-causing ability of *S.* Typhimurium. However, the required involvement of flagella in the pathogenesis of *Salmonella* raises the concern that, with only one phase of flagellar antigens being expressed, *S.* 4,[5],12:i:- may have an impaired ability to infect swine and cause disease[16-18].

Only two studies have been published to date on the pathogenesis and severity of disease caused by *S.* 4,[5],12:i:- infections in swine, and of those studies, contradictory findings have been reported. One study determined that *S.* 4,[5],12:i:- does not induce a fever following experimental infection of 7-week old pigs[19] while the other reported that a significant increase in rectal temperature occurs by the second day post infection (DPI)[20]. In the first study, *S.* Typhimurium and *S.* 4,[5],12:i:- were both reported as being capable of causing diarrhea in weaned pigs, and although the timing of the diarrhea varied with serovar, both serovars caused diarrhea seven or more days following inoculation[19]. This is also in contrast to the second study which reported that *S.* 4,[5],12:i:- infection of swine led to diarrhea by DPI 2[20]. Neither study assessed the gross or histologic pathology associated with inoculation of *S.* 4,[5],12:i:- in swine, although a retrospective study found that identification of *S.* 4,[5],12:i:- from clinical specimens was associated with histopathologic evidence of disease[21]. While little is known about *S.* 4,[5],12:i:-, the literature is much more clear on the fact that *S.* Typhimurium is considered to be pathogenic in swine while other common serovars such as *S.* Derby are not know to display significant pathogenicity despite frequent isolation[19,21,22]. Given the differences in the presentation of salmonellosis based on the infecting serovar as well as inter-study differences, more work is needed, with larger sample sizes of pigs and various stages of growth to better determine the effect of *S.* 4,[5],12:i:- infection in swine, particularly when compared to other serovars with known levels of pathogenicity.

The ability to colonize tissues throughout the pig has also been shown to differ by serovar. While *S.* Typhimurium is able to colonize the ileum, ileocecal lymph nodes, tonsils, and mandibular lymph nodes, other serovars such as *S.* Rissen and *S.* Anatum have been shown to colonize a smaller subset of those tissues[23]. However, *S.* Typhimurium is less able to colonize non-enteric viscera (liver, spleen, lung) and skeletal muscles when compared to intestines, colon, tonsils, and mesenteric lymph nodes[24]. Similarly, mesenteric lymph nodes collected from swine at the time of slaughter proved to be frequently positive for *Salmonella* of various serovars, including *S.* 4,[5],12:i:-, although they were predominantly positive for *S.* Typhimurium[25,26]. It remains unclear based on this study whether the observed difference in prevalence of serovars in the mesenteric lymph nodes of swine was due to variations in the prevalence of serovars infecting swine or in the ability of each of the serovars to colonize tissues throughout swine. Following experimental infections with *S.* 4,[5],12:i:-, the tonsils, ileocecal lymph nodes, cecal mucosa, and Peyer's patches were all colonized with *Salmonella* on 7 DPI in one study[20] while the tonsils, mesenteric lymph nodes, and intestinal tissues were observed to be colonized with *Salmonella* on 21 and 49 DPI in another study[19]. Due to the strong correlation between colonization of various tissues of swine at the time of slaughter and increased risk of contamination of the carcass[23], it is necessary to understand the colonization potential of each serovar and the role of host factors to fully understand the potential risks from *Salmonella* infections.

Yet another risk associated with *Salmonella* infections of swine is the potential for persistence of the organism, with subsequent transmission to other pigs or contamination of their environment through shedding in feces[27-29]. With *Salmonella* transmission occurring primarily through the fecal-oral route, an improved awareness of the expected shedding pattern would also

facilitate appropriate interpretation of fecal culture results and understanding of the course of infection. Many serovars have been documented to cause persistent infections in swine as evidenced by prolonged fecal shedding, including Typhimurium, Derby, Yoruba, and Cubana[30,31], although the duration of persistence varies with serovar, infecting dose, and host-specific factors[2,30]. In pigs naturally infected with various serovars of *Salmonella*, fecal shedding was highly variable on an individual pig basis in terms of both the pattern and amount of shedding[2]. Only one study has been completed to evaluate the persistence of *S.* 4,[5],12:i:- in swine following a known infection event. *Salmonella* 4,[5],12:i:- has been detected in the feces of all four swine 49 days after infection with *S.* 4,[5],12:i:-[19], indicating the potential to cause persistent infections although more work is needed given that only four pigs were maintained in that study until DPI 49.

The rise in prevalence of *Salmonella* 4,[5],12:i:- in livestock and humans also raises the question of why the serovar has recently emerged and become increasingly prevalent. *Salmonella* 4,[5],12:i:- has repeatedly been reported to be more highly resistant to antimicrobials relative to *S.* Typhimurium[7,32,33], which may provide a significant advantage to its survival in swine operations with extensive antimicrobial drug use. Likewise, *S.* 4,[5],12:i:- is commonly resistant to heavy metals such as zinc and copper, which can be added to livestock feeds as an alternative to antimicrobial drugs to reduce bacterial infections[15,34]. With the antimicrobial properties of some heavy metals, it is evident why resistance to them would provide a selective advantage over more susceptible serovars. Another potential mechanism leading to the emergence of the *S.* 4,[5],12:i:- serovar is the ability to outcompete other serovars *in vivo.* *Salmonella* 4,[5],12:i:-, despite lacking one phase of flagellar antigens, has retained its ability to adhere to and invade porcine intestinal epithelial cells *in vitro*[35]. Additionally, a study of 133

monophasic isolates showed that the majority possessed the ability to form biofilms[36]; this could enhance the survivability while reducing the effects of antimicrobials on the bacteria[36,37]. *Salmonella* 4,[5],12:i:- also commonly possesses multiple virulence genes that may contribute to its survival within the host and environment; these genes include but are not limited to *sipC* which is involved in cell adhesion and invasion, *sopB* which is involved in the intestinal changes that lead to diarrhea, and *hilA* which activates the invasion process[38]. The combination of biofilm formation, presence of virulence genes involved in the pathogenesis, resistance to antimicrobials, and resistance to heavy metals may all function together to provide a selective and competitive advantage to *S.* 4,[5],12:i:-.

Based on the limited data available, we hypothesized that *Salmonella* 4,[5],12:i:- possesses abilities similar to that of *S.* Typhimurium and greater than that of *S.* Derby in regards to disease-causing ability, colonization, and persistence in swine. We also hypothesized that *S.* 4,[5],12:i:- displays a competitive advantage in colonization over *S.* Typhimurium to allow the monophasic serovar to predominate in swine. To address some of the gaps in knowledge related to these hypotheses, three separate animal studies were performed with the goal of answering the following questions: 1) determine the clinical course of disease caused by *S.* Typhimurium, *S.* 4,[5],12:i:-, and *S.* Derby relative to uninfected pigs; 2) compare the ability of the three serovars to colonize tissues throughout the pig; 3) evaluate the persistence of the three serovars in swine over time; and 4) compare the ability of *S.* 4,[5],12:i:- to outcompete *S.* Typhimurium in co-inoculated pigs.

Materials and Methods

Salmonella isolate selection

Salmonella enterica serovars Typhimurium, 4,[5],12:i:-, and Derby isolates were selected from the collection of clinical isolates submitted to the Iowa State University (ISU) Veterinary Diagnostic Laboratory (VDL). These isolates were originally cultured from clinical samples submitted to the ISU-VDL using standard laboratory protocols (see CHAPTER 2). Serotyping was completed by the National Veterinary Services Laboratory (NVSL). Selection of isolates for all studies was based on the following criteria: 1) isolation from clinical samples submitted to the ISU-VDL, 2) originate from 3-13 week old pigs, and 3) association with histopathologic lesions suggestive of salmonellosis. For the pathogenesis study (animal study #2), an isolate of *S.* Typhimurium that had previously been used in a successful animal study (ISU-VDL, unpublished data) that met all of the criteria above was selected to be used. To ensure identification of an appropriate clinical isolate of *Salmonella* 4,[5],12:i:- that had retained its virulence following laboratory passage, a small scale preliminary study was performed using three separate isolates of 4,[5],12:i:- meeting the above criteria (animal study #1). For the competitive fitness animal study (animal study #3), clinical isolates of *S.* Typhimurium and *S.* 4,[5],12:i:- that met the above criteria and exhibited complimentary resistance profiles (S. 4,[5],12:i:- isolate susceptible to ceftiofur but resistant to gentamicin; *S.* Typhimurium isolate susceptible to gentamicin but resistant to ceftiofur) were utilized for this study. Antimicrobial susceptibility testing was completed using the TREK Sensititre™ system and Sensititre™ Bovine/Porcine MIC Plate (ThermoFisher Scientific, Catalog #BOPO6F) according to Clinical and Laboratory Standards Institute (CLSI) guidelines in the VET08 document. Ceftiofur has a veterinary-specific swine breakpoint for respiratory disease pathogens only (*Streptococcus suis*,

Actinobacillus pleuropneumoniae, and *Pasteurella multocida*) from which the breakpoint for *Enterobacteriaceae* is currently extrapolated. Gentamicin has human *Enterobacteriaceae* breakpoints that can be utilized to guide breakpoint determinations in swine. Based on the human and veterinary breakpoints available for these antimicrobials, extrapolated breakpoints were used to determine susceptibility, intermediate, and resistance for the antibiotics of interest on the Sensititre™ Bovine/Porcine MIC Plate.

General culture conditions, inoculum preparation, and sample culture

Salmonella isolates from clinical cases submitted to the ISU-VDL were stored in brain heart infusion (BHI) broth with 20% glycerin at -80°C. Isolates were removed from the freezer, sub-cultured onto tryptic soy agar with 5% sheep blood (Remel Products, Lenexa, KS), and incubated for 18-24 hours at 35°C. Plates were evaluated for purity of culture prior to use.

Inoculum preparation for in vivo studies

Once pure cultures were obtained, colonies of *Salmonella* were added to Mueller Hinton (MH) broth (BD Diagnostics, Sparks, MD). Based on quantitative *Salmonella* culture completed previously to determine the correlation between the optical density and CFUs of *Salmonella*, a target optical density (OD600) of 0.09 would approximately correlate to $1x10^8$ CFU/mL; this was the goal of the inoculum. Once the inoculum was prepared, serial dilutions of the inoculum were plated to Tryptic Soy Agar with 5% sheep blood (Remel Products) to determine the actual concentration of *Salmonella*, and the remaining inoculum was stored in the refrigerator until administered to the piglets.

Salmonella detection and quantification

Fecal and tissue samples were collected during the animal studies for culture using both quantitative and enrichment techniques. All samples collected for culture, including feces throughout the trial and tissues from necropsy, were stored in the refrigerator (4˚C) immediately following collection and were then transferred to the freezer (-80˚C) within 5 hours of collection until further processing could be completed.

Samples collected for quantification of *Salmonella* were thawed at 37˚C until they reached room temperature, weighed, and added to Phosphate Buffered Saline (PBS) (Fisher Scientific, Rochester, MN) to create a 1:10 dilution of each sample. Tissue samples were further ground with a mortar-and-pestle type grinder to facilitate pipetting of samples. Serial dilutions were plated to Xylose-Tergitol-Lysine-4 (XLT4) agar (Remel Products) and then incubated at 35°C without CO_2. Colonies with morphology characteristic of *Salmonella* were counted daily for three days. Interpretation of standard plate count results used the following criteria: 1) quantification at day three was used to calculate concentration in the original sample unless plates were overgrown with normal flora at day three in which case counts from previous days were used to calculate concentrations; 2) plates with 25-250 colonies were considered reliably countable and 3) counts were averaged if more than one plate was in the countable range. Samples that had *Salmonella* detected by quantitative culture but below the countable level of 25-250 colonies were listed as 750 CFU/mL. Samples that had *Salmonella* detected by enrichment culture but not from quantitative culture were listed as 500 CFU/mL. A minimum of one characteristic and representative colony per sample per pig was confirmed as *Salmonella* using Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry (MALDI-TOF-MS) following manufacturer's recommendations (Bruker Daltonic Inc., Billerica, MA). Per

ISU-VDL protocol, a minimum MALDI-TOF-MS confidence score of 2.10 was required for a confirmatory genus level identification.

In addition to quantification by serial dilutions, 0.25 mL of 1:10 dilution of each sample was enriched in 5 mL of Buffered Peptone Water (BPW) (Remel Products). The BPW was incubated for 18-24 hours at 35°C without CO_2 prior to subculture to Brilliant Green agar with Novobiocin (BGN) (BD Diagnostics; Sigma-Aldrich, St. Louis, MO) and XLT4 agar. The BGN and XLT4 agars were incubated at 35°C without CO_2 for 48 hours and were observed for colonies with morphology characteristic of *Salmonella*. A minimum of one colony from enrichment subculture per sample was confirmed as *Salmonella* using MALDI-TOF-MS.

For isolation and identification of each serovar of *Salmonella* following co-inoculation of both Typhimurium and 4,[5],12:i:- from samples in animal study #3, *Salmonella* quantification was performed via standard plate counts on XLT4 supplemented with either ceftiofur or gentamicin [3 types of XLT4 agar: 1) XLT4 agar with gentamicin sulfate (VetOne, Boise, ID) at a concentration of 8 ug/mL to inhibit growth of *S.* Typhimurium, 2) XLT4 agar with ceftiofur in the form of Naxcel® (Zoetis, Parsippany, NJ) at a concentration of 0.5 ug/mL to inhibit growth of *S.* 4,[5],12:i:-, and 3) XLT4 without additional antibiotics]. To ensure the plates were inhibitive to the expected serovar, 1-2 representative colonies were confirmed as *Salmonella* by MALDI-TOF-MS and then were identified at the serovar level using PCR (see CHAPTER 2).

Animal studies

General information

All studies involving animals were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC) prior to initiation (11-16-8391-S). All swine used in

the studies were five weeks of age at the initiation of the study; this age was selected based on evaluation of the most common age of animals positive for 4,[5],12:i:- from diagnostic samples from pigs with diarrhea and histologic lesions consistent with salmonellosis submitted to the ISU-VDL over the past several years as well as successful induction of disease with other serovars of *Salmonella* in this age group[27,39,40]. All animals were pre-screened as negative for Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and Porcine Epidemic Diarrhea Virus (PEDV) via pooled polymerase chain reaction (PCR) testing as well as *Salmonella* negative status via individual enrichment fecal culture and/or enriched PCR prior to initiation of the study. The pigs were acclimated for 72 hours following arrival prior to inoculation during which time baseline weights, temperatures, and fecal scores were recorded and pre-inoculation fecal swabs were obtained. Pigs were randomly assigned to treatment groups, and each treatment group was housed in separate biosecure rooms for the duration of each study. Throughout the acclimation and study periods, all pigs were fed a complete diet of non-medicated corn and soybean meal except for the 12 hours prior to inoculation during which all pigs were held off feed. All pigs were euthanized using barbiturate overdose.

Scoring systems: fecal consistency, gross pathology, and histopathology

Fecal scoring was standardized across all trials on a scale of 1-5 (1 = dry feces, 2 = moist feces, 3 = mild diarrhea, 4 = severe diarrhea, and 5 = watery diarrhea) as previously described[41]. This scoring system is depicted in Figure 1. Fecal scores of 1 and 2 were both considered to be normal scores, with a score of 2.5 or above indicating the presence of diarrhea. Pigs were diagnosed with clinical disease when the fecal score was 3 or above and/or rectal temperature outside of the normal range of 101.5-103.5˚F.

Gross necropsy scoring was completed on animals across all trials in a standardized manner. The severity and distribution of gross lesions were observed along the intestinal tract, with the entire intestinal tract length opened and evaluated for the presence of fibrinous exudate and necrotic portions. Samples collected at time of necropsy for histopathologic evaluation were placed in 10% neutral buffered formalin and included the following: liver; spleen; ileocecal lymph nodes; proximal, middle, and distal jejunum; ileum; cecum; middle and apex of spiral colon; and rectum. Histologic evaluation was performed at the ISU-VDL by a pathologist who was blinded to the pig numbers and treatment groups. The histologic evaluation protocol is summarized in Table 1. The ileocecal lymph node, spleen, and liver were evaluated for the presence or absence of neutrophils in 5-400X fields of view. These tissues were scored on a scale of 1-6 (1=neutrophils absent in all views, 6=neutrophils present in all views). The mean neutrophil count from 5-400X fields of view was determined for each small and large intestine sample. Any view with more than 100 neutrophils was considered “too numerous to count” and listed as 100 for averaging purposes. For large intestine samples, the mean was obtained of three crypt depths per section measured at 10X. Lastly, an ulceration score was determined for each small and large intestine sample. The ulceration score was equal to the number of crypts over which the most severe foci extended. This score ranged from 0 to 5, with 0 indicating a lack of observed ulceration and 5 indicating an ulcer spanning 5 or more crypts.

A scoring system was derived to obtain an overall score of the histopathology data to facilitate comparison between sections of intestine. For a mean neutrophil count of less than 5, 0 points were assigned. For every increase by 10 neutrophils, one additional point was assigned (i.e. 1 point for 5-10 neutrophils, 2 points for 11-20 neutrophils, 3 points for 21-30 neutrophils, etc.). Ulceration scores translated directly as points to the overall score (i.e. ulceration score of 2

added 2 points to the overall score). For crypt depths, the scoring system was as follows: <700 um = 0 points, 700-800 um = 1 point, 800-900 um = 2 points, 900-1000 = 3 points, >1000 um = 4 points). The crypt depth score was shifted to the right by 100 um for the rectum due to longer crypts in health (i.e. <800 = 0 points, 800-900 = 1 point, etc.). One additional point was added if evidence of submucosal inflammation was noted. Another point was added if crypt abscesses were observed.

Animal study #1: Preliminary evaluation of pathogenicity of Salmonella 4,[5],12:i-

To identify an appropriate clinical isolate of *Salmonella* 4,[5],12:i:- that had retained its pathogenic ability following laboratory passage, a small scale pilot study was performed using three separate isolates of 4,[5],12:i:-, isolates ISU-SAL0239-15 (A), ISU-SAL0240-15 (B), and ISU-SAL0241-16 (C), meeting the above criteria. A total of nine five-week old pigs were individually identified and randomly assigned to one of three treatment groups with three pigs per isolate. Each group was then orally inoculated with 10 mL of approximately 5 x 10^8 CFU/mL *Salmonella* inoculum with one of the three clinical isolates of *S.* 4,[5],12:i:-. The actual concentration of the inoculum was 5.3 x 10^7 CFU/mL, 6.9 x 10^7 CFU/mL, and 8.6 x 10^7 CFU/mL for *S.* 4,[5],12:i:- isolates ISU-SAL0239-15 (A), ISU-SAL0240-15 (B), and ISU-SAL0241-16 (C), respectively. The course of clinical disease as indicated by rectal temperature, fecal scoring, fecal *Salmonella* quantification was then followed for seven days. After seven days, the animals were euthanized and necropsies were performed to evaluate for gross and histopathologic signs of disease. Tissue samples collected at necropsy for *Salmonella* quantification included liver, spleen, tonsils, and ileocecal lymph nodes. The results of this study

were utilized to select an isolate of *S.* 4,[5],12:i:- to optimize the results of the large-scale animal study presented below.

Animal study #2: Pathogenicity of Salmonella Typhimurium, 4,[5],12:i:-, and Derby in swine

To determine the ability of *Salmonella* serovar 4,[5],12:i:- to cause disease and establish a carrier state in swine, 72 five-week old pigs were utilized in a month-long study examining the effect and duration of infection with 4,[5],12:i:- when compared to the known highly pathogenic serovar Typhimurium and lesser pathogenic serovar Derby. The pigs were individually identified and randomly assigned to the following treatments: 1) 20 pigs received oral inoculation with 4,[5],12:i:- only (isolate ISU-SAL240-15), 2) 20 pigs received oral inoculation with Typhimurium only (isolate ISU-SAL243-14), 3) 20 pigs received oral inoculation with Derby only (isolate ISU-SAL242-16), and 4) 12 sham-inoculated pigs to serve as negative control. The pigs were housed in pens of four, with five total groups per treatment and three for the control group. The groups for each serovar were housed in separate rooms to ensure no cross contamination between treatments would occur.

Following acclimation, the pigs were inoculated with a standardized dose for all serotypes of 10 mL of 1 x 10^8 CFU/mL *Salmonella* utilizing a combination of 8 mL oral gavage and 2 mL swabbed directly in the back of the mouth ensuring tonsil exposure as occurs during natural infection with *Salmonella*. The actual inoculum concentrations were 1.44 x 10^8 CFU/mL, 1.53 x 10^8 CFU/mL, and 1.94 x 10^8 CFU/mL for *S.* 4,[5],12:i:-, *S.* Derby, and *S.* Typhimurium, respectively. Daily fecal scores were taken on all animals for the first seven days to monitor progression of clinical disease; as overt clinical disease was expected to decrease after the first

week of infection, bi-weekly fecal scores were taken for the remainder of the study. All pigs had rectal temperatures recorded for the first seven days and bi-weekly thereafter for the remainder of the study. Fecal samples were collected from the rectum of all pigs at 2 DPI, and all pigs still alive at DPI 4, 7, 14, 21 and 28 for quantitative and enriched *Salmonella* fecal culture to determine the amount of shedding of *Salmonella* into the environment over time following infection.

On DPI 2 and 4, five pigs per treatment group and three control pigs were assigned to be euthanized for tissue collection based on severity of clinical signs (i.e. *Salmonella*-infected pigs demonstrating the most severe clinical signs based on a combination of rectal temperature and fecal score). The remaining pigs after day 4 (10 per experimental group; 6 in control group) were allowed to complete the study and were euthanized for sample collection at 28 DPI. At the time of euthanasia, evaluation of gross lesions was completed and samples were collected for histopathologic evaluation of the jejunum, ileum, cecum, colon, ileocecal lymph nodes, tonsils, liver, and spleen to evaluate the progression of clinical disease over time. Additional tissue samples, including ileocecal lymph nodes, tonsils, liver, spleen, and colon contents, were collected at the time of necropsy for quantitative *Salmonella* culture to assess the level of *Salmonella* colonization in these tissues at various points over time following inoculation.

Two pigs were removed from this study. One pig was in the *S.* Derby group and was removed from the study due to premature death on DPI 2. The pig was submitted to the ISU VDL and determined to have died from a vitamin E-selenium responsive nutritional cardiomyopathy, better known as Mulberry Heart Disease. The other pig removed from the study was among the control group pigs euthanized on DPI 2 and was removed from the study due to

the presence of *Isospora suis*, a primary pathogen of swine, detected during histopathologic evaluation. All other animals completed the study and were included in the analysis.

Animal study #3: Competitive fitness of Salmonella Typhimurium and 4,[5],12:i:- in swine

To determine if *Salmonella* 4,[5],12:i:- has the ability to outcompete other pathogenic serovars of *Salmonella*, such as Typhimurium, *in vivo*, 12 five-week old pigs were co-inoculated with a 50:50 mixture of the two serovars. Six additional pigs, three for *S.* Typhimurium and three for *S.* 4,[5],12:i:-, were singly inoculated for a controlled comparison. The isolates used for the study were *Salmonella* 4,[5],12:i:- ISU-SAL245-16 and *Salmonella* Typhimurium ISU-SAL244-16. The co-inoculated pigs were housed in pens of four, with three total groups in a single room, while each of the singly inoculated groups were housed in a single pen of three each in separate rooms. Following acclimation, the pigs were inoculated with a standardized dose of 10 mL of 1 x 10^8 CFU/mL *Salmonella* utilizing combination of 8 mL oral gavage and 2 mL swabbed directly in the back of the mouth for each serovar, for a total of 20 mL for co-inoculated and 10 mL for singly inoculated animals. The actual inoculum concentrations were 1.38 x 10^8 CFU/mL and 1.73 x 10^8 CFU/mL for *S.* Typhimurium and *S.* 4,[5],12:i:-, respectively. Following inoculation, daily rectal temperatures and fecal scores were taken on all animals for the first seven days to monitor progression of clinical disease; bi-weekly temperatures and fecal scores were taken for the remainder of the study. Fecal samples were collected from all pigs alive on DPI 1-5, 7 and 10 for quantitative culture of both serovars of *Salmonella*.

One pig that was inoculated with both *S.* Typhimurium and *S.* 4,[5],12:i:- died on DPI 3. This pig was submitted for evaluation at the ISU VDL following the same protocol as described

for the study. It was determined that the pig had developed septicemia with both *Salmonella* serovars as the ileum, colon, liver, spleen, tonsils, and ileocecal lymph nodes were all culture positive for *Salmonella*. One additional pig was euthanized on DPI 6 due to neurologic deficits, severe fever, and deteriorating condition. This pig was also submitted for evaluation at the ISU VDL, and was determined to have meningoencephalitis caused by *Haemophilus parasuis*. Both of these pigs were removed from the analysis, leaving 10 pigs in the co-infected group. On DPI 4, five random co-inoculated pigs and all six of the singly inoculated pigs were euthanized, and then tonsils and ileocecal lymph nodes were collected. The remaining five pigs were euthanized on DPI 10 with collection of tonsils and ileocecal lymph nodes.

The competition index (CI) was calculated using the following formula: $(X - Y)/(X + Y)$, in which X is the number of *Salmonella* 4,[5],12:i:- colonies and Y is the number of *Salmonella* Typhimurium colonies[42]. A CI value that is positive indicates that *Salmonella* 4,[5],12:i:- is more fit while a value that is negative indicates that *Salmonella* Typhimurium is more fit. A CI value closer to 1 or -1 indicates dominance of *Salmonella* 4,[5],12:i:- or *Salmonella* Typhimurium, respectively.

Statistical analysis

No statistical analysis was completed for animal study #1 as the goals of the study could be accomplished without statistical analysis. For the data sets from animal studies #2 and #3, the population homogeneity was assessed using the Shapiro-Wilk test and the population variance was assessed using the Brown-Forsythe test. Based on the results from these two tests, either parametric or non-parametric tests were chosen. For brevity, the same statistical test was used for each dependent variable when necessary (i.e. temperature, quantitative culture). For quantitative

culture of feces from animal study #2, one-way ANOVA followed by Dunnett's test was used to compare the mean amount of *Salmonella* on a $\log_{10}$ basis on DPI 0 to the mean amount of *Salmonella* on all other days, within each serovar. For the rectal temperatures from animal study #2, the Kruskal-Wallis test with Dunn's post test was utilized to compare the mean temperature on each DPI to the day of inoculation (DPI 0) within each serovar. Rectal temperatures, fecal scores, and histologic lesion scores were also compared between serovar groups at each DPI using the GLIMMIX procedure of the SAS System to complete the Tukey-Kramer test. For quantitative fecal culture from animal study #3, Poisson distribution was used to compare *S.* Typhimurium to *S.* 4,[5],12:i:- at each DPI. For all tests, a P-value less than 0.05 was deemed significant.

Results

Animal study #1

Results of the preliminary animal study comparing three separate isolates of *Salmonella* 4,[5],12:i:- demonstrated that clinical disease, as indicated by a rectal temperatures outside of the normal range (101.5-103.5˚F) (Figure 2) and/or diarrhea (fecal score greater than or equal to 2.5) (Figure 3), was induced by all isolates, although isolates A and B caused an increase in rectal temperature and in fecal score while isolate C caused an increase in the fecal score only. Fecal scores and temperatures from the three isolates, averaged among each group, reached their peak at DPI 3 and 2, respectively. There was detectable shedding of *Salmonella* in the feces with direct culture throughout the entire seven-day study in piglets inoculated with isolates A and B. For the group inoculated with isolate C, mild clinical disease was noted with *Salmonella* shedding only detectable through DPI 4 in pre-enrichment feces. However, *Salmonella* was

detectable in enriched fecal samples from all pigs at all time points for all groups inoculated with each of the three isolates (Table 2).

Ileocecal lymph nodes, tonsils, spleen, and liver were collected at the time of necropsy on DPI 7, with culture results listed in Table 2. The spleen and liver proved to be a poor sample type for the detection of *Salmonella* 4,[5],12:i:- infections as only one out of nine and two out of nine pigs, respectively, were positive for *Salmonella* by quantitative and/or enrichment culture. Ileocecal lymph nodes and tonsils proved to be a better sample for detection of *Salmonella* 4,[5],12:i:- as nine out of nine and seven out of nine pigs, respectively, were positive for *Salmonella* by direct and/or enrichment culture. Although *Salmonella* 4,[5],12:i:- was detectable in the feces and tissues of the majority of pigs, only one out of nine pigs had gross lesions suggestive of salmonellosis, which consisted of fibrinous colitis; this pig was inoculated with isolate B. Histopathologic evaluation of intestinal tissues on the basis of neutrophil infiltration, crypt elongation, and ulceration revealed that the cecum and spiral colon were the primary target of *Salmonella* 4,[5],12:i:-, regardless of the specific isolate involved in infection, with minimal to no lesions in the small intestine and rectum (Figure 4). From this combination of data, isolate B of *S.* 4,[5],12:i:- was selected for the full scale animal study described below.

Animal study #2

Clinical disease

Based on rectal temperature and fecal scoring, overall, *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:- both appeared to cause a similar short-lived clinical disease that was more severe than that caused by *S.* Derby infection. The rectal temperature results are shown in Figures 5A-5D. Those infected with *S.* 4,[5],12:i:- had a peak mean temperature on DPI 2 at

102.7˚F (± 0.3), but this was not significantly different from DPI 0 until DPI 5, 6, 7, 10, and 17 (Figure 5A). The mean temperature (± SE) of pigs infected with *S.* Typhimurium peaked on DPI 2 at 103.2˚F (± 0.3), with a statistically significant increase relative to DPI 0 on DPI 1, 2, and 4 (Figure 5B). *Salmonella* Derby-inoculated pigs developed the highest mean temperature on DPI 1 at 103.1˚F (± 0.1), with a significant difference from DPI 0 on DPI 1 only (Figure 5C). The control pigs had a significantly elevated temperature compared to their DPI 0 mean on DPI 5, 6, and 10 (Figure 5D). Interestingly, there were a number of animals with temperatures that were both significantly higher and lower than normal in the *S.* Typhimurium and *S.* 4,[5],12:i:- groups, but not the control or *S.* Derby groups. Thus, calculation of the average temperature in each group may not be the best measure of clinical disease manifestation in these groups as severely ill animals can also exhibit lower than normal body temperatures. For example, on DPI 1, 2, 3, and 4, 25%, 45%, 27%, and 87%, respectively, of *S.* 4,[5],12:i:- infected pigs had rectal temperatures outside the normal range of 101.5-103.5˚F. This is in contrast to 0%, 18%, 0%, and 22% for control pigs over the same time period.

The fecal score results are shown in Figure 6. The mean fecal score (± SE) of *S.* Typhimurium-infected pigs peaked on DPI 2 at 3.7 (± 0.3) and *S.* 4,[5],12:i:- -infected pigs peaked on DPI 2 at 3.5 (± 0.3). The *S.* Derby infected pigs reached a mean fecal score on DPI 4 of 2.8 (± 0.2) with all other mean fecal scores being at or below 2.4, therefore indicating that *S.* Derby did not successfully induce diarrhea in the pigs with the exception of DPI 4. The sham-inoculated control pigs reached a peak fecal score on DPI 28 at 3.5 (± 0.2), but also had elevated mean fecal scores on DPI 10 at 3.3 (± 0.2) and DPI 3 at 2.9 (± 0.4). *Salmonella* 4,[5],12:i:- had a significantly increased mean fecal score relative to *S.* Derby and *S.* Typhimurium pigs on DPI 0. The mean fecal score of *S.* 4,[5],12:i:- pigs was significantly increased relative to *S.*

Typhimurium on DPI 1 and *S.* Derby and the controls on DPI 2. The mean fecal score of *S.* Typhimurium pigs was also significantly increased relative to *S.* Derby and the controls on DPI 2. *Salmonella* 4,[5],12:i:- pigs had a significantly higher mean fecal score relative to the controls on DPI 4.

Bacterial culture

All pre-inoculation fecal samples were negative for *Salmonella* culture. Throughout the duration of the study, all samples collected from the control pigs were confirmed negative for *Salmonella* by enrichment culture. Fecal culture results are listed in Table 3 and depicted in Figure 7. The mean amount of *Salmonella*, on $\log_{10}$ basis of colony forming units (CFU) per 0.5 grams of feces, peaked at 3.0 (± 0.1) on DPI 4 in *S.* Typhimurium infected pigs. Pigs infected with *S.* 4,[5],12:i:- reached a peak level of *Salmonella* in feces on DPI 2 at 3.4 (± 0.2) with those with *S.* Derby also reached a peak level on DPI 2 at 2.9 (± 0.1). All serovar groups had a significantly increased amount of *Salmonella* in the feces on DPI 2, 4, and 7 relative to DPI 0, and *S.* Typhimurium also had a significantly increased amount in the feces on DPI 14. Enriched feces remained positive for *Salmonella* in the *S.* Typhimurium group in 20% of pigs (2 of 10) on DPI 28, while *S.* 4,[5],12:i:- only remained positive until DPI 21, at which time 30% of fecal samples (3 of 10) were positive.

Samples were also collected for culture from pigs at necropsy on DPI 2, 4, and 28. Culture results of samples collected at necropsy are listed in Table 4. On DPI 2 and 4, all *Salmonella*-infected pigs, with the exception of one *S.* Derby-infected pig on DPI 4, had detectable levels of *Salmonella* in their colon contents. The liver and spleen of *Salmonella*-infected pigs had variable results based infecting serovar and timing after inoculation, ranging

from 60% of *S.* Typhimurium pigs positive in the liver on DPI 2 while *S.* Derby inoculated pigs were negative for *Salmonella* in the spleen and liver at all necropsy time points. The ileocecal lymph nodes were positive on DPI 2 and 4 from all *Salmonella*-infected pigs. However, the lymph nodes were only positive in 50% (5 of 10) of *S.* 4,[5],12:i:- pigs, 67% (6 of 9) of *S.* Derby pigs, and 40% (4 of 10) of *S.* Typhimurium pigs on DPI 28. Tonsils were positive from all *Salmonella*-infected pigs on DPI 2 and 4, except for three *S.* Derby pigs on DPI 4 and one *S.* Typhimurium pig on DPI 2. On DPI 28, tonsils were positive for *Salmonella* from 90% (9 of 10) of *S.* 4,[5],12:i:- pigs, 67% (6 of 9) of *S.* Derby pigs, and 40% (4 of 10) of *S.* Typhimurium pigs.

Gross lesions

Gross lesions suggestive of salmonellosis were absent in all control pigs and *S.* Derby infected pigs necropsied on DPI 2 but were present in two out of five *S.* Typhimurium pigs and two out of five *S.* 4,[5],12:i:- pigs. At DPI 4, similar results were found, with two out of five *S.* 4,[5],12:i:- pigs and four out of five *S.* Typhimurium pigs possessing gross lesions suggestive of salmonellosis while none of the control or *S.* Derby pigs had gross lesions. Representative gross lesions from *S.* 4,[5],12:i:- and *S.* Typhimurium pigs on DPI 4 are shown in Figure 8A and 8B, respectively. However, by DPI 28, only one *S.* Typhimurium pig and one *S.* 4,[5],12:i:- pig had gross lesions suggestive of salmonellosis.

Histopathology

In alignment with the histopathologic results from study #1 of three *S.* 4,[5],12:i:- isolates, ulceration, neutrophil infiltration, and crypt elongation were primarily limited to the cecum and spiral colon for all serovars (Figure 9). On DPI 2 (Figure 9A), there were statistically

significant differences in histologic lesion scores in the ileum, cecum, and spiral colon. In the ileum, *S.* 4,[5],12:i:- had significantly more severe lesions than control and *S.* Derby pigs but not more severe than *S.* Typhimurium. In the cecum and mid spiral colon, *S.* Typhimurium and *S.* 4,[5],12:i:- had significantly more severe lesions than control and *S.* Derby but not more severe than one another. In the apex of the spiral colon, *S.* Typhimurium had significantly more severe lesions than *S.* 4,[5],12:i:-, control, and *S.* Derby, but *S.* 4,[5],12:i:- lesions were not significantly different from those of *S.* Derby or control. On DPI 4 (Figure 9B), there were statistically significant differences in the histologic lesion scores in the cecum and mid spiral colon only. In the cecum, *S.* 4,[5],12:i:- had significantly more severe lesions than *S.* Typhimurium, *S.* Derby, and controls, but *S.* Typhimurium did not possess significantly more severe lesions than *S.* Derby or controls. In the mid spiral colon, *S.* Typhimurium and *S.* 4,[5],12:i:- had significantly more severe lesions than control and *S.* Derby but not more severe than one another. On DPI 28 (Figure 9C), there were no statistically significant differences in the histologic scores between the treatment groups in any of the intestinal sections evaluated. Overall, histopathologic lesions suggestive of clinical salmonellosis were present consistently in the *S.* Typhimurium and *S.* 4,[5],12:i:- groups on DPI 2 and 4 but not the *S.* Derby or control groups. *Salmonella* 4,[5],12:i:- infection resulted in a similar severity of diarrhea, disturbance in rectal temperature, colonization of tissues, and gross and histologic lesions as with *Salmonella* Typhimurium infection.

Balantidium coli, a secondary pathogen, was noted upon histologic examination in a portion of pigs from all groups euthanized on DPI 2 (5/5 4,[5],12:i:- pigs, 1/5 Typhimurium pigs, 1/5 Derby pigs, and 0/2 control pigs), 4 (5/5 4,[5],12:i:- pigs, 3/5 Typhimurium pigs, 2/5 Derby pigs, and 0/3 control pigs), and 28 (1/10 4,[5],12:i:- pigs, 2/10 Typhimurium pigs, 0/9 Derby pigs, and 0/3 control pigs), respectively. *Cryptosporidium* was also detected on DPI 2 (in 2/5

4,[5],12:i:- pigs, 1/5 Typhimurium pig, 2/5 Derby pigs, and 1/2 control pigs) and on DPI 4 (in 3/5 4,[5],12:i:- pigs, 1/5 Typhimurium pig, 3/5 Derby pigs, and 2/3 control pigs). No *Cryptosporidium* was detected on DPI 28. The presence of *Cryptosporidium* did not appear to cause a severe increase in the fecal scores, rectal temperatures, or histopathologic scores in any of the treatment groups as the majority of these measurements were either equivalent between pigs that were positive and negative for *Cryptosporidium* or higher in those that were negative for *Cryptosporidium*. No other lesions suggestive of clinical disease caused by other pathogens of swine were detected during the histopathologic examination.

Animal study #3

To ensure that both the *S.* Typhimurium and *S.* 4,[5],12:i:- isolates were able to cause disease when infecting a pig individually, three pigs were inoculated with only *S.* Typhimurium and three with only *S.* 4,[5],12:i:-. In *S.* 4,[5],12:i:- infected pigs, the mean temperature peaked on DPI 2 at 102.5˚F. *Salmonella* Typhimurium infected pigs had a peak mean temperature of 102.4˚F on DPI 4. The peak mean fecal scores of each group were 3.7 on DPI 3 in *S.* 4,[5],12:i:- infected pigs and 3 on DPI 2 and DPI 4 in *S.* Typhimurium infected pigs. These results together indicate that both isolates were able to individually cause mild clinical disease in pigs. In pigs that received both *S.* Typhimurium and *S.* 4,[5],12:i:-, the mean fecal score peaked on DPI 1 at 4.1, but remained above 3.5 on DPI 2, 3, and 4. The mean temperature of co-infected pigs peaked at 103.3˚F on DPI 2. There was a notable increase in the severity and duration of clinical disease in pigs infected simultaneously with *S.* Typhimurium and *S.* 4,[5],12:i:- compared to pigs infected with only one serovar of *Salmonella*.

In the 10 pigs that completed the co-inoculation study, there was a higher mean amount of *S.* 4,[5],12:i:- detected via culture compared to *S.* Typhimurium at DPI 1, 2, 3, 4, 5, and 10, and the difference was statistically significant on DPI 1, 2, 3, 4, and 10 (Figure 10). The largest difference in amounts of *S.* 4,[5],12:i:- and *S.* Typhimurium occurred on DPI 2. On DPI 7, the only time point in which the average amount of *S.* Typhimurium exceeded the amount of *S.* 4,[5],12:i:- in feces, the difference was not statistically significant. In addition, when compared to singly infected pigs, co-infected pigs shed *Salmonella* at higher levels on average. Of the three pigs infected with *S.* 4,[5],12:i:- and four samples collected from each pig on DPI 1-4, only three out of 12 samples had detectable levels of *Salmonella* present in their feces. Similar results were found with the *S.* Typhimurium pigs, with only three out of 12 samples possessing detectable levels of *Salmonella*.

All three pigs infected with *S.* Typhimurium were positive for *Salmonella* in their tonsils and ileocecal lymph nodes on DPI 4. Of the three pigs infected with *S.* 4,[5],12:i:-, only one had *Salmonella* present in the tonsils on DPI 4, and two had *Salmonella* in the ileocecal lymph nodes. This is in contrast to those pigs that were infected simultaneously with both *S.* Typhimurium and *S.* 4,[5],12:i:-, in which seven pigs had tonsils culture positive for *S.* 4,[5],12:i:- and six pigs positive for *S.* Typhimurium (Figure 11). Of those pigs positive for *Salmonella* in their tonsils, five had higher levels of *S.* 4,[5],12:i:-, two had higher levels of *S.* Typhimurium, and 3 had equivalent levels of the two serovars. As for the ileocecal lymph nodes, eight pigs were positive for *S.* 4,[5],12:i:- and six were positive for *S.* Typhimurium. Similar to the tonsils, five had higher levels of *S.* 4,[5],12:i:-, one had higher levels of *S.* Typhimurium, and four had equivalent levels of the two serovars. The competition index (CI) was calculated for each set of samples collected. In the feces on all days except for DPI 7, the competition index was greater than zero,

indicating that *S.* 4,[5],12:i:- has a higher level of fitness compared to *S.* Typhimurium (Figure 12A). Upon necropsy on DPI 4 (Figure 12B), the CI was greater than zero (0.32 and 0.39 in the tonsils and ileocecal lymph nodes, respectively). Upon necropsy on DPI 10 (Figure 12B), the CI was also greater than zero (0.52 and 0.20 in the tonsils and lymph nodes, respectively). These results indicate that *S.* 4,[5],12:i:- also demonstrated a higher level of fitness in colonization of tonsils and ileocecal lymph nodes when compared to *S.* Typhimurium.

Discussion

The work completed in these studies clearly demonstrates that *Salmonella* serovar 4,[5],12:i:- possesses a similar ability as *Salmonella* Typhimurium to cause significant clinical disease in swine. In addition, this serovar, along with Typhimurium and Derby, can be carried in the tonsils and lymph nodes and shed in the feces of infected animals for weeks following exposure and illness. Potential sequelae to this carriage includes likely contamination of the environment with subsequent infection of pen mates and contamination of carcasses and meat at harvest leading to food safety concerns. Overall, all three animal studies provided insight into the pathogenesis of disease caused by *S.* 4,[5],12:i:- in swine. Animal study #1 allowed us to compare three separate isolates of *S.* 4,[5],12:i:-, all of which had retained their ability to cause disease through the culture process as indicated by the observed fever and/or diarrhea. Fecal shedding for all isolates continued for the duration of the study from DPI 1-7.

Animal study #2 enabled evaluation of the pathogenicity, fecal shedding, and colonization of swine by *S.* Typhimurium, *S.* 4,[5],12:i:-, and *S.* Derby. The mean rectal temperature of pigs infected with all three *Salmonella* serovars peaked at DPI 1-2 The mean fecal score reached its peak at DPI 2 for *S.* Typhimurium and *S.* 4,[5],12:i:-, with *S.* Derby

reaching a lower peak at a later time than the other two serovars. The rectal temperatures and fecal scores indicated that *S.* 4,[5],12:i:- has a similar disease-causing ability to *S.* Typhimurium and induces a more severe disease than that caused by *S.* Derby. Fecal shedding continued through DPI 28 for *S.* Typhimurium and DPI 21 for *S.* 4,[5],12:i:- and *S.* Derby. The colonization of tonsils and ileocecal lymph nodes appeared to be similar across DPI 2, 4, and 28 in all three serovar groups. Gross lesions suggestive of Salmonellosis were concentrated to DPI 2 and 4 and were limited to those pigs infected with *S.* Typhimurium or *S.* 4,[5],12:i:-. Histologic lesions occurred primarily in the cecum and spiral colon, with significantly higher scores in the pigs infected with *S.* Typhimurium and *S.* 4,[5],12:i:-.

Animal study #3 revealed that co-infection with *S.* 4,[5],12:i:- and *S.* Typhimurium simultaneously may cause a more severe clinical disease compared to infection with only one serovar, as indicated by the more severe fever and diarrhea in co-infected pigs relative to singly-infected pigs. It is unclear if the increased disease severity in co-infected pigs was the result of a synergistic effect of the two serovars or the higher inoculum dose in co-infected pigs. The *S.* 4,[5],12:i:- isolate demonstrated a competitive advantage of *S.* Typhimurium *in vivo*, which is evidenced by the significantly higher mean levels of *S.* 4,[5],12:i:- relative to *S.* Typhimurium on DPI 1, 2, 3, 4, and 10.

Many studies have demonstrated that *S.* 4,[5],12:i:- is a monophasic variant of *S.* Typhimurium based on pulsed-field gel electrophoresis (PFGE)[11,43,44], multiple-locus variable analysis (MLVA)[8,45], polymerase chain reaction (PCR)[10,14,46], phage typing[11,44,47,48], and whole-genome sequencing[49,50]. However, the characterization of *S.* 4,[5],12:i:- as a pathogen of swine has not been as readily documented. To our knowledge, only two studies have been published on experimental infections of swine with *S.* 4,[5],12:i:-, leaving a large gap in our understanding of

this emerging serovar. One of the recently published studies was a small-scale study in experimentally infected swine, which revealed that *S.* 4,[5],12:i:- has maintained a similar disease-causing ability to that typically associated with *S.* Typhimurium[20]. Specifically, at DPI 2, the infected pigs developed a fever and diarrhea, and they shed *Salmonella* 4,[5],12:i:- in their feces throughout the seven days following inoculation[20]. Overall, the authors of this study concluded that both *S.* Typhimurium and its monophasic variant, *S.* 4,[5],12:i:-, can cause gastrointestinal disturbances, further substantiating the claim that expression of only one flagellar phase does not alter the pathogenicity[20,35]. The results of this small-scale study with respect to the clinical disease established by *S.* 4,[5],12:i:- was in alignment with our work as we determined that diarrhea and fever occur on DPI 2 following infection with *S.* 4,[5],12:i:- and that the disease and lesions induced by *S.* 4,[5],12:i:- closely resemble that induced by *S.* Typhimurium. However, that study utilized fecal moisture content to determine if the pigs had developed diarrhea[20]. Fecal moisture content analysis is a much more objective way to evaluate the level of diarrhea in swine compared to fecal scoring, even though fecal scoring is a more common method in studies of this type[41,51]. Due to the size and scope of our study, we utilized fecal scoring to facilitate evaluation of a larger sample size.

The other study that was recently completed was similar to our comparison of *Salmonella* serovars 4,[5],12:i:-, Typhimurium, and Derby[19]. In contrast to our findings, this study found that all *S.* 4,[5],12:i:-, *S.* Derby, and *S.* Typhimurium infected pigs, with the exception of one *S.* Derby pig on DPI 21, one *S.* Typhimurium pig on DPI 14, and one *S.* Typhimurium pig on DPI 45, were shedding *Salmonella* throughout the study at all time points from DPI 1-49[19]. This is contrary to the findings of our study and other studies that have reported that *Salmonella* shedding varies on an individual basis, varies with the infecting serovar, and is not

continuous[2,28-30]. The deviation in this study from what has been reported previously from other studies and our findings is likely due to the larger amount of feces collected for culture, as this study used 30 grams of feces and an increased fecal sample mass has been correlated to increased sensitivity[52]. This study also reported that *S.* 4,[5],12:i:- infection of swine resulted in fever and diarrhea on DPI 21 while diarrhea observed in pigs infected with *S.* Typhimurium occurred on DPI 7 and 10 and with *S.* Derby on DPI 14[19]. This is much different from that observed in other studies of experimental infections with *S.* Typhimurium and *S.* 4,[5],12:i:- in swine, which generally report fever and diarrhea on DPI 2-4[20,53,54], as well as our study which also indicated disease occurs much sooner after infection.

Salmonella Typhimurium is well-recognized as an enteric pathogen of swine. Many studies have been completed to determine the course of disease typical of the pathogen. It caused significantly increased rectal temperatures and fecal scores in three to four week old pigs at DPI 2-3 while also colonizing the liver, spleen, tonsils, and ileocecal junction[53]. Another study echoed similar findings in four-week old experimentally infected pigs, with 100% of pigs shedding *Salmonella* in their feces through DPI 28, 20% developing a fever (>103˚F), and 55% exhibiting diarrhea[55]. Both of these studies came to similar conclusions to the conclusions that were reached following our animal study #2: *S.* Typhimurium causes fever, diarrhea, and fecal shedding of the organism following infection. *Salmonella* Derby has been less thoroughly evaluated *in vivo* in swine, likely due to its lesser-pathogenicity. However, one study found that it is shed in the feces of some pigs through DPI 56 and potentially even longer, indicating an ability to persist within the host[30]. The results from our studies showed *S.* Derby is shed for a shorter duration in the feces, specifically through DPI 21, compared to the study that reported fecal shedding of *S.* Derby occurs for 8 weeks post infection. Given the presence of *Salmonella*

in the tissues collected on DPI 28 from all three groups, however, including *S.* Derby, it is clear that *Salmonella* is able to persist within infected pigs through DPI 28. The absence of *Salmonella* in feces was likely the result of low diagnostic sensitivity in our study and/or a low quantity of *Salmonella* being shed rather than an absence of infection.

A subset of pigs in animal study #2 developed a fever while another subset developed hypothermia. When comparing the mean rectal temperatures of the pigs in each treatment group, it was noted that *S.* 4,[5],12:i:- did not appear to successfully induce a fever. However, evaluation of the rectal temperatures of the pigs by the percentage within, above, or below the normal temperature range (101.5-103˚F) revealed that *S.* 4,[5],12:i:- caused a fever in some and hypothermia in others. Specifically, in the first four days following infection, *S.* 4,[5],12:i:- had 25-90% of pigs with rectal temperatures outside of the normal range, which was comparable to *S.* Derby pigs with 11-50% outside of the normal range and *S.* Typhimurium pigs with 20-35% outside of the normal range. This is in contrast to the control pigs in which no more than 22% of pigs had rectal temperatures outside of the normal range during the same timeframe. Fever is widely accepted as a response to infection, as it creates a more hostile environment for the bacteria within the host to improve host resistance to spread of the infection. However, hypothermia represents another potential response to infection. Hypothermia is a mechanism in place thought to downregulate pro-inflammatory cytokine release to reduce excessive tissue damage and is generally associated with severe systemic infection[56,57]. Oddly, the control pigs as well as the *S.* 4,[5],12:i:- also exhibited as a group decreased body temperatures between DPI 5-7; as the whole group experienced this change, it is likely that room temperature fluctuations contributed to this changes in these groups. All four experimental groups were housed in

separate rooms throughout the study which may explain why this was not seen in the other two groups.

When co-inoculated at the same levels, *S.* 4,[5],12:i:- was consistently detected in the feces of a higher percentage of pigs and at higher levels than *S.* Typhimurium in our study. Additionally, the competition index showed that *S.* 4,[5],12:i:- has a greater level of fitness in the host than *S.* Typhimurium. This finding may partly explain why *S.* 4,[5],12:i:- has been increasingly identified in swine diagnostic samples over the past several years. Additional data suggests that co-infection may promote enhanced clinical disease in swine. Although no studies have been published on the clinical disease resulting from simultaneous infections with more than one serovar of *Salmonella*, there have been reports of simultaneous infections of *Salmonella* and other viral or bacterial pathogens of swine having additive effects[58,59].

Identification of co-infections is likely rare in practice as only a single colony of *Salmonella* is typically selected from the culture plate for final identification and characterization[60,61], leaving the possibility for co-infections to be occurring frequently yet rarely detected. Future research is warranted to better understand the impact that co-infections might play in clinical disease in the field. Our results showed a potential synergism between *S.* 4,[5],12:i:- and *S.* Typhimurium in swine, but a larger sample size and equivalent inoculum concentrations between co-infected and singly-infected groups would be necessary to confirm this.

Our studies were not completed without limitations. All samples collected for culture were frozen at -80˚C from the time of collection to the time of processing up to two months later. The freezing and thawing process may have reduced the viability of some of the *Salmonella* initially present in the samples[62]. Ideally, all samples would have been processed within several

hours of collection without any freezing; unfortunately, the large volume of samples collected at any given time point made this option less feasible. An alternative to culture is PCR testing. Many PCR tests have been validated for the detection and identification of *Salmonella*[63-65], which would have also been able to quantify the *Salmonella* present in the samples, however, this method was not cost-effective for the large number of samples collected during this study. An additional culture-related limitation was the relatively small amount of feces used for culture. The diarrhea induced by the *Salmonella* infection created challenges regarding collection of large amounts of feces from the rectum of each pig, therefore, a lesser amount of starting material was used that might have been ideal to increase the sensitivity of the test.

In an effort to increase the odds of detecting acute gross and histologic lesions induced by *Salmonella* infections, *Salmonella*-infected pigs in animal study #2 were selected for euthanasia on DPI 2 and 4 based on severity of clinical disease. That is, pigs with the highest combined fecal score and rectal temperature in each pen were selected for euthanasia. There is known to be significant individual-level variation in the effects of *Salmonella* infections in pigs, with some pigs even failing to develop clinical signs, gross lesions, and/or histologic lesions suggestive of infection. If there is a correlation between the severity of clinical disease and persistence of infection or colonization of tissues, these measurements could be biased. The method of selection utilized on DPI 2 and 4 likely also increased the mean gross and histologic lesion scores on these days while potentially decreasing the mean scores on DPI 28. It also may have decreased the mortality rate following *Salmonella* infections. However, it enabled characterization of the location and types of lesions induced by *Salmonella*.

During the histopathologic evaluation of tissues from animal study #2, *Cryptosporidium* and *Balantidium coli* were noticed from a portion of the pigs in all serovar groups and the control

group at all euthanasia time points. The presence of *Cryptosporidium* did not appear to cause a severe increase in the fecal scores, rectal temperatures, or histopathologic scores in any of the treatment groups. This is evidenced by the fact that, in a comparison of the mean fecal scores, rectal temperatures, and histopathologic scores, the majority of measurements were either equivalent between pigs that were positive and negative for *Cryptosporidium* or higher in those that were negative for Cryptosporidium. Cryptosporidium can cause a mild and self-limiting diarrhea in pigs, although it generally causes an asymptomatic infection[66], so its effect on the pigs in this study remains unknown. *Cryptosporidium* was not one of the pathogens that the pigs were screened for prior to arrival, so it is unclear when the infection was obtained. While it would be most ideal to screen the pigs for all possible pathogens including *Cryptosporidium* prior to enrollment in the study, it would be an inefficient use of resources; therefore, the pathogens of the highest concern were selected for screening. *Balantidium coli* was also noted in the intestinal sections of some pigs. However, this is not generally considered to be a primary pathogen[67] and was likely not independently the cause of diarrhea in the pigs. The fecal scores and rectal temperatures of the control group were mildly increased in the last 1-2 weeks of animal study #2, which could have been due to infection by a non-*Salmonella* pathogen. Further testing of samples for other pathogens was not pursued, however, given the lack of histopathologic evidence of the presence of other pathogens of concern.

A potential confounder of animal study #3 was the difference in the inoculum dose provided to singly-infected pigs and co-infected pigs. All pigs were infected with the same dose of each serotype, which was 1.38 x 10^8 CFU/mL for those receiving *S.* Typhimurium and 1.73 x 10^8 CFU/mL for those receiving *S.* 4,[5],12:i:-. The co-infected pigs received two doses of inoculum though as they were infected with a full dose of *S.* 4,[5],12:i:- and a full dose of *S.*

Typhimurium, resulting in twice as much *Salmonella* in the inoculum as that received by the singly-infected pigs. However, this is a comparatively small difference, with singly-infected pigs receiving just 0.35 $\log_{10}$ CFU/g less *Salmonella* at most than co-infected pigs. If this study were to be repeated, administration of the same total dose of *Salmonella* in the inoculum for singly and co-infected pigs would aid in differentiation of the effect of the dose from the effect of the co-infection.

In conclusion, our results clearly indicate that *S.* 4,[5],12:i:- induces clinical disease comparable to that of *S.* Typhimurium with similar corresponding gross and histopathologic lesions. The cause of the emergence of the monophasic serovar may be due in part to the competitive advantage *S.* 4,[5],12:i:- may possess *in vivo*, as evidenced by the higher mean levels of *S.* 4,[5],12:i:- relative to *S.* Typhimurium in the majority of pigs infected with the two serovars simultaneously. Future research should focus on assessing the frequency and potential synergistic effects of concurrent *S.* 4,[5],12:i:- and *S.* Typhimurium infections in swine.

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Tables

Table 1. Summary of histopathologic measurements obtained from each of the evaluated tissue sections during all three animal studies.

Histopathologic Evaluation	
Tissue Location	**Obtained Measurements**
Proximal jejunum Mid jejunum Distal jejunum Ileum	Neutrophil count* Ulceration score†
Cecum Apex of spiral colon Mid spiral colon Rectum	Neutrophil count* Crypt depth‡ Ulceration score†
Ileocecal lymph node Spleen Liver	Neutrophil presence§

FOV = field of view; TNTC = too numerous to count

*Average of 5 neutrophil counts; at 400X FOV, >100 neutrophils in one FOV was considered TNTC and listed as 100

†Number of crypts over which most severe ulceration foci extended, ranging from 0-5 (5 = ulcer spanning five or more crypts)

‡Average of three crypt depths measured at 10X FOV

§Presence or absence of neutrophils (in any amount) in 5-400X FOV, ranging from 1-5 (1 = no neutrophils observed in 5 FOV, 5 = neutrophils observed in all 5 FOV)

Table 2. Culture results of three separate isolates of *Salmonella* 4,[5],12:i:- from animal study #1.

	Culture positives†						
Inoculum	**Fecal Samples (DPI)**			**Necropsy Samples***			
	2	**4**	**7**	**Liver**	**Spleen**	**IC LN**	**Tonsils**
***S*. 4,[5],12:i:- (A)**	100%	100%	100%	33%	0%	100%	100%
	3/3	3/3	3/3	1/3	0/3	3/3	3/3
***S*. 4,[5],12:i:- (B)**	100%	100%	100%	0%	0%	100%	33%
	3/3	3/3	3/3	0/3	0/3	3/3	1/3
***S*. 4,[5],12:i:- (C)**	100%	100%	100%	33%	33%	100%	100%
	3/3	3/3	3/3	1/3	1/3	3/3	3/3

DPI = days post inoculation; IC LN = ileocecal lymph node

*Necropsy samples were collected on DPI 7

†Positives were positive for *Salmonella* from quantitative culture and/or enrichment culture

Table 3. Comparison of *Salmonella* culture results in feces collected from pigs inoculated with *Salmonella* serovars 4,[5],12:i:-, Typhimurium and Derby in animal study #2.

	Fecal culture positives*					
Inoculum	**DPI**					
	2	**4**	**7**	**14**	**21**	**28**
S. 4,[5],12:i:-	100%	73%	90%	40%	30%	0%
	20/20	11/15	9/10	4/10	3/10	0/10
S. Typhimurium	95%	93%	60%	40%	20%	20%
	19/20	14/15	6/10	4/10	2/10	2/10
S. Derby	95%	79%	67%	11%	11%	0%
	18/19	11/14	6/9	1/9	1/9	0/9

DPI = days post inoculation

*Positives were positive for *Salmonella* from quantitative and/or enrichment culture

Table 4. Comparison of *Salmonella* culture results from samples collected during necropsy from pigs inoculated with *Salmonella* serovars 4,[5],12:i:-, Typhimurium and Derby in animal study #2

Inoculum	Necropsy DPI	Culture Positives* Location				
		Colon	**Liver**	**Spleen**	**IC LN**	**Tonsils**
S. 4,[5],12:i:-	**2**	100% 5/5	40% 2/5	20% 1/5	100% 5/5	100% 5/5
	4	100% 5/5	40% 2/5	20% 1/5	100% 5/5	100% 5/5
	28	10% 1/10	0% 0/10	0% 0/10	50% 5/10	90% 9/10
S. Derby	**2**	100% 5/5	0% 0/5	0% 0/5	100% 5/5	100% 5/5
	4	80% 4/5	0% 0/5	0% 0/5	100% 5/5	40% 2/5
	28	0% 0/9	0% 0/9	0% 0/9	67% 6/9	67% 6/9
S. Typhimurium	**2**	100% 5/5	60% 3/5	20% 1/5	100% 5/5	80% 4/5
	4	100% 5/5	20% 1/5	0% 0/5	100% 5/5	100% 5/5
	28	30% 3/10	0% 0/10	0% 0/10	40% 4/10	40% 4/10

DPI = days post inoculation; IC LN = ileocecal lymph nodes

*Positives were positive for *Salmonella* from quantitative and/or enrichment culture

Figures

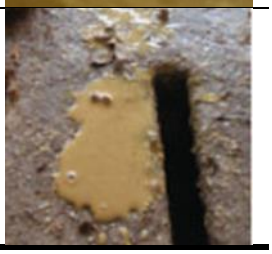
Fecal Score	Description	Image
1	Firm and dry	
2	Firm and moist	
3	"Cow-pie" appearance; increasingly moist with no clumping on digital rectal exam	
4	"Pancake" appearance; increasingly moist, flattens out on floor	
5	Consistency resembles water	

Figure 1. Depiction and explanation of fecal scoring system utilized for all three animal studies to determine the level of diarrhea.

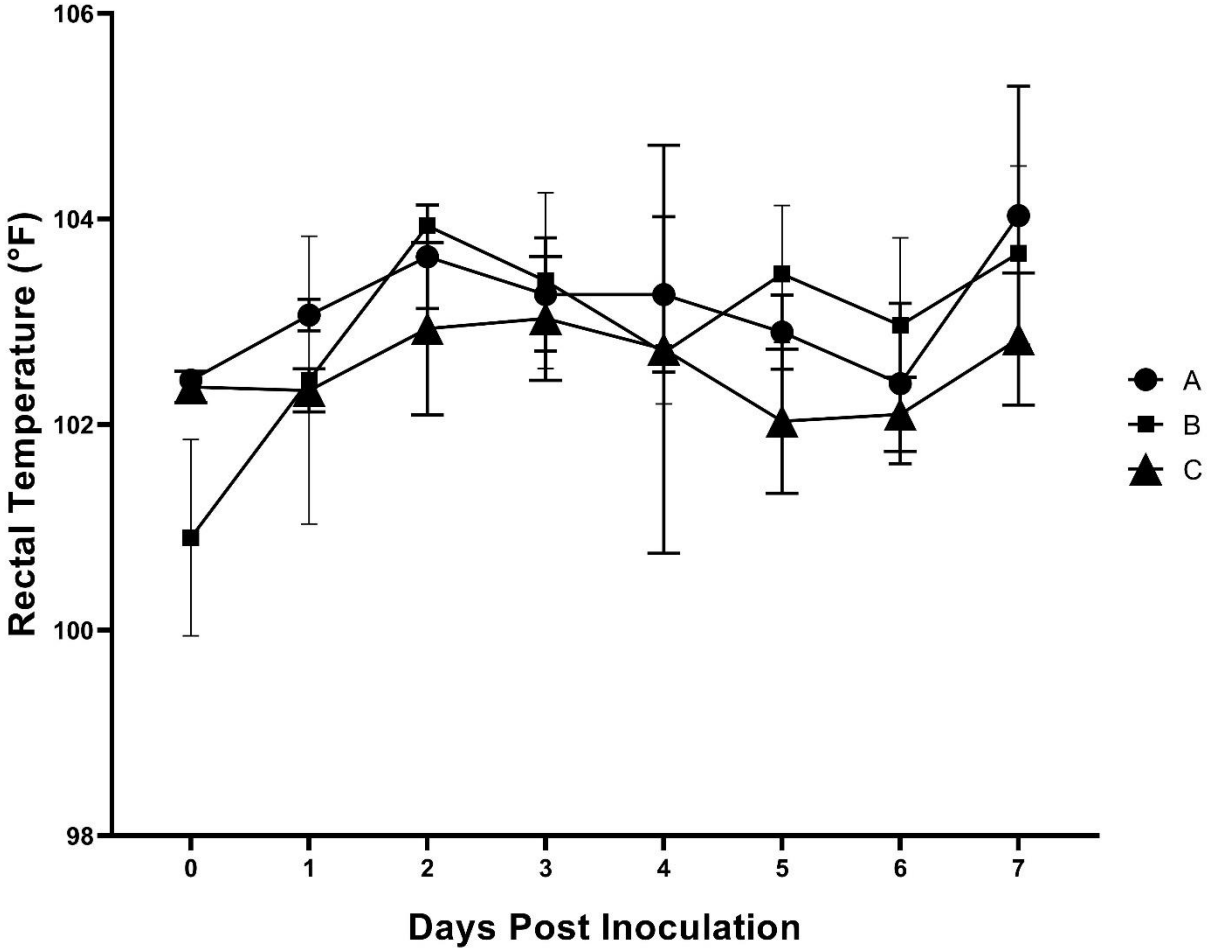


Figure 2. Mean rectal temperatures from animal study #1 following inoculation on DPI 0 with three separate isolates of *S.* 4,[5],12:i:- (A, B, and C). The mean and standard error are represented by the bars.

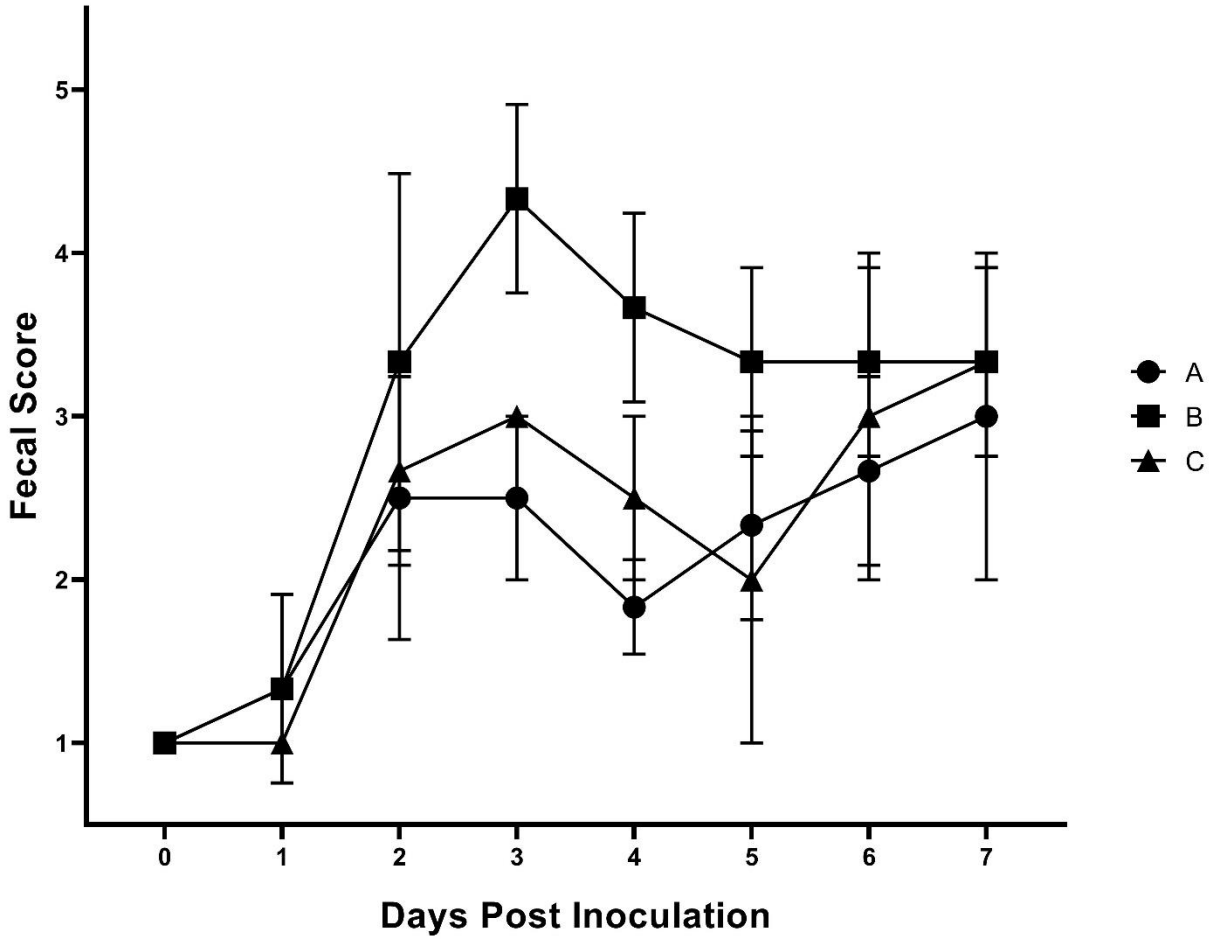


Figure 3. Mean fecal scores from animal study #1 following inoculation on DPI 0 with three separate isolates of *S.* 4,[5],12:i:- (A, B, and C). The fecal scoring system ranged from 1-5, with 1-2 being considered normal and 5 being considered severe diarrhea. The mean and standard error are represented by the bars.

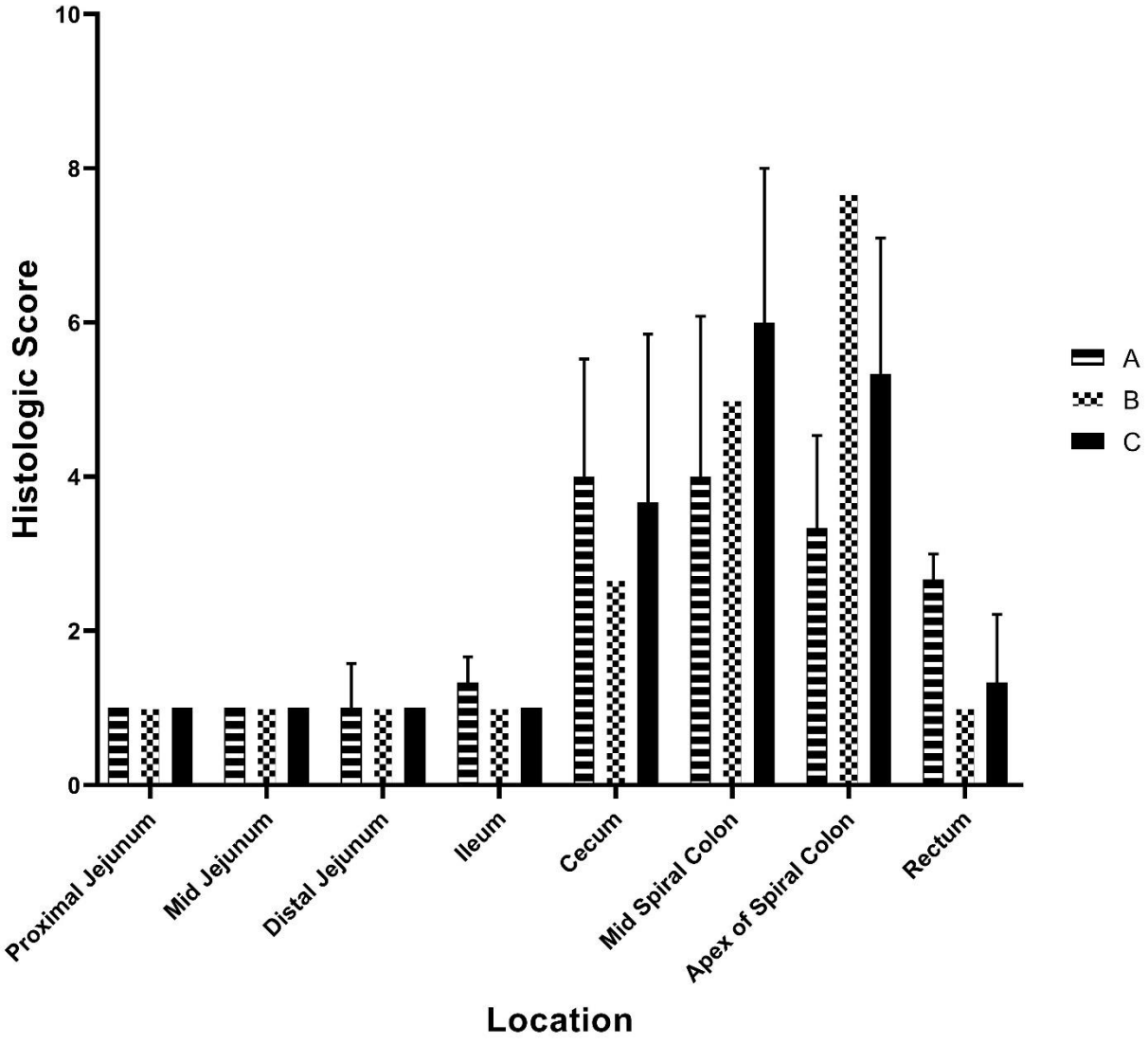
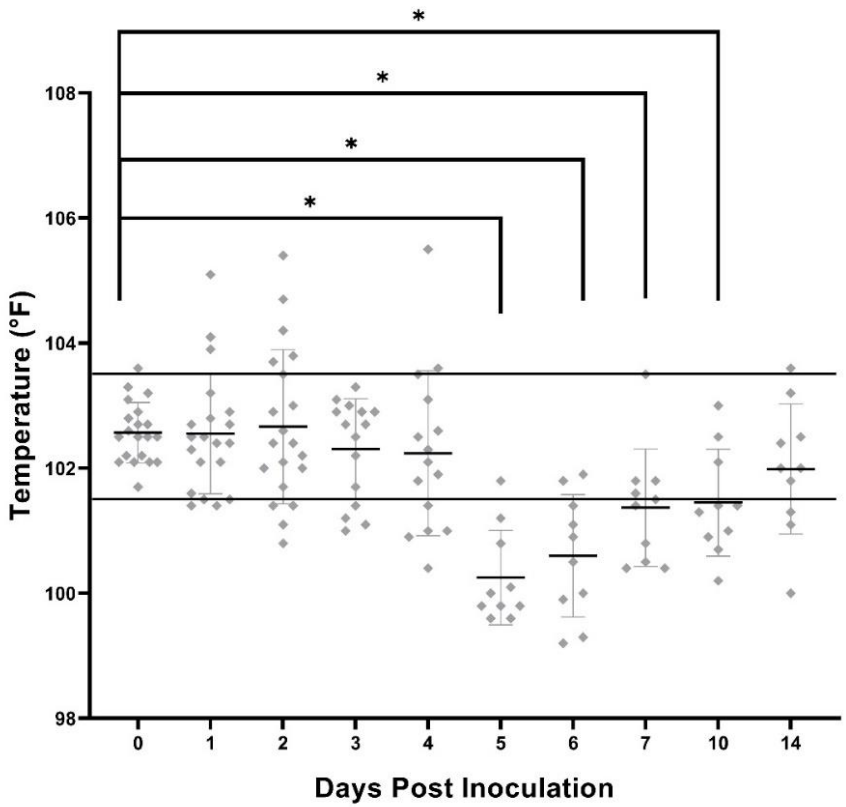


Figure 4. Histologic scores from samples collected at necropsy on day seven following inoculation with three separate isolates of *S.* 4,[5],12:i:- (A, B, and C) in animal study #1. These scores depict the average histologic lesions, as determined by the ulceration, neutrophil infiltration, and crypt elongation and abscessation, and submucosal inflammation, at the time of necropsy on DPI 7. The mean of each isolate-tissue location combination is represented by the bar with the standard error represented by the line.



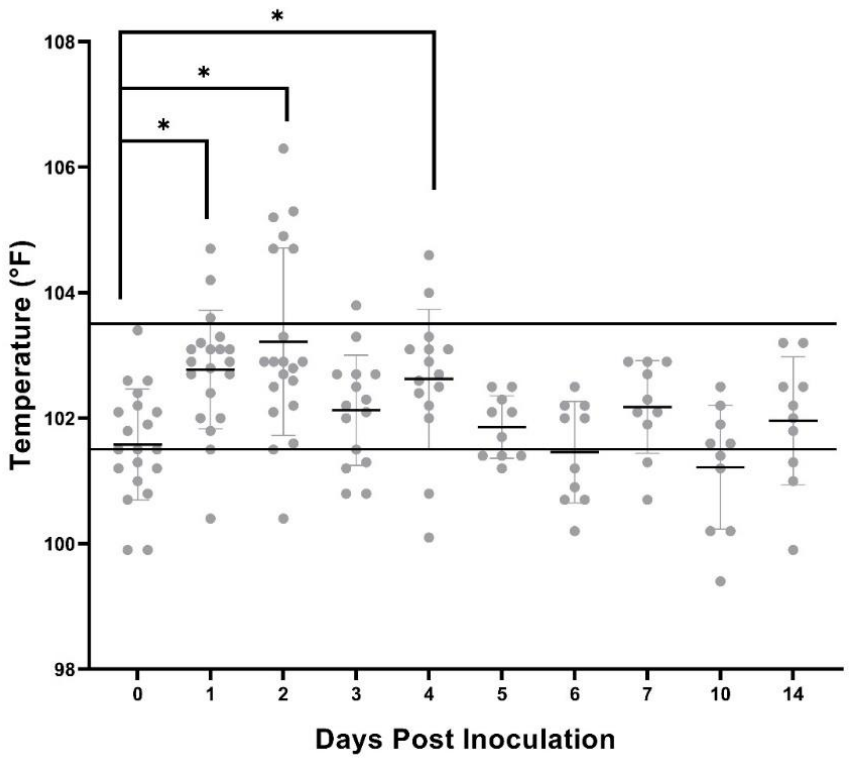


Figure 5A. **Figure 5B.**

Figure 5. Comparison of rectal temperatures of pigs inoculated with *Salmonella* serovars (5A) 4,[5],12:i:-, (5B) Typhimurium, and (5C) Derby and (5D) non-inoculated control pigs in animal study #2. Asterisks represent significant differences from the mean temperature on DPI 0 ($p<0.05$). Each datum point represents the temperature in individual animal; short horizontal bar represents mean temperature within a group; long horizontal bars located at 101.5˚F and 103.5˚F, between which is the normal temperature range for swine.

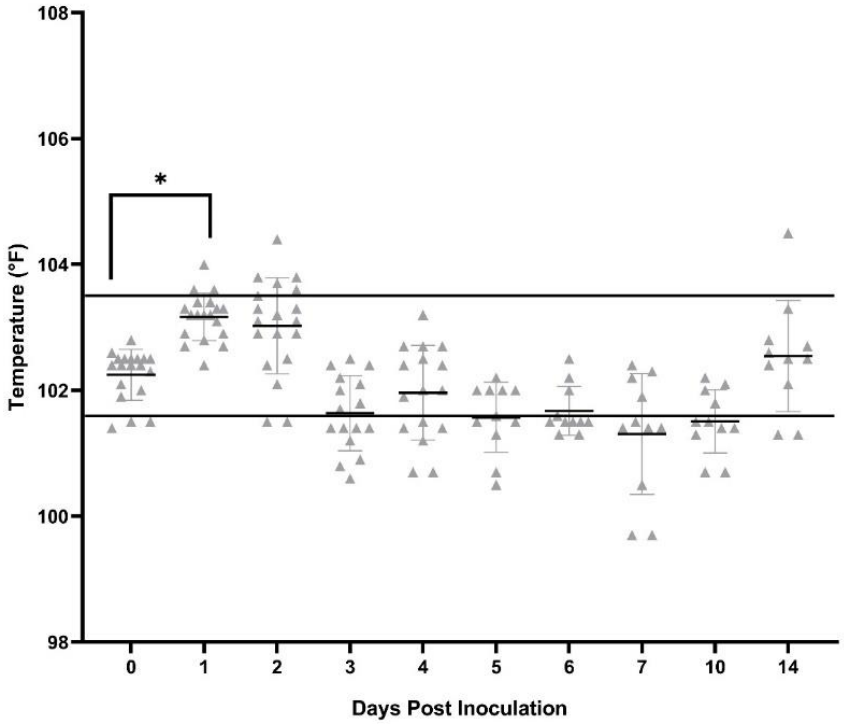


Figure 5C. (continued)

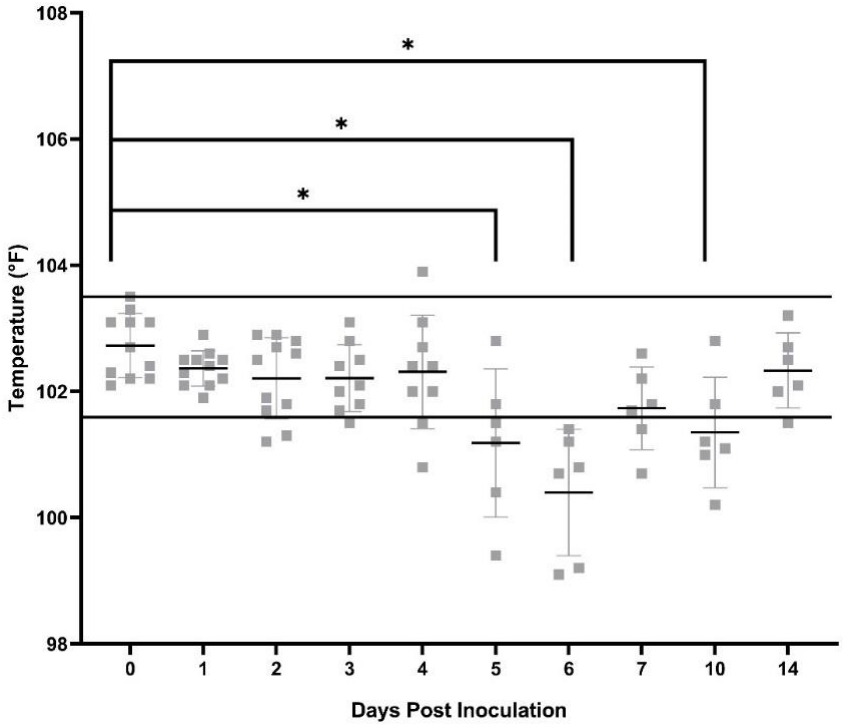


Figure 5D. (continued)

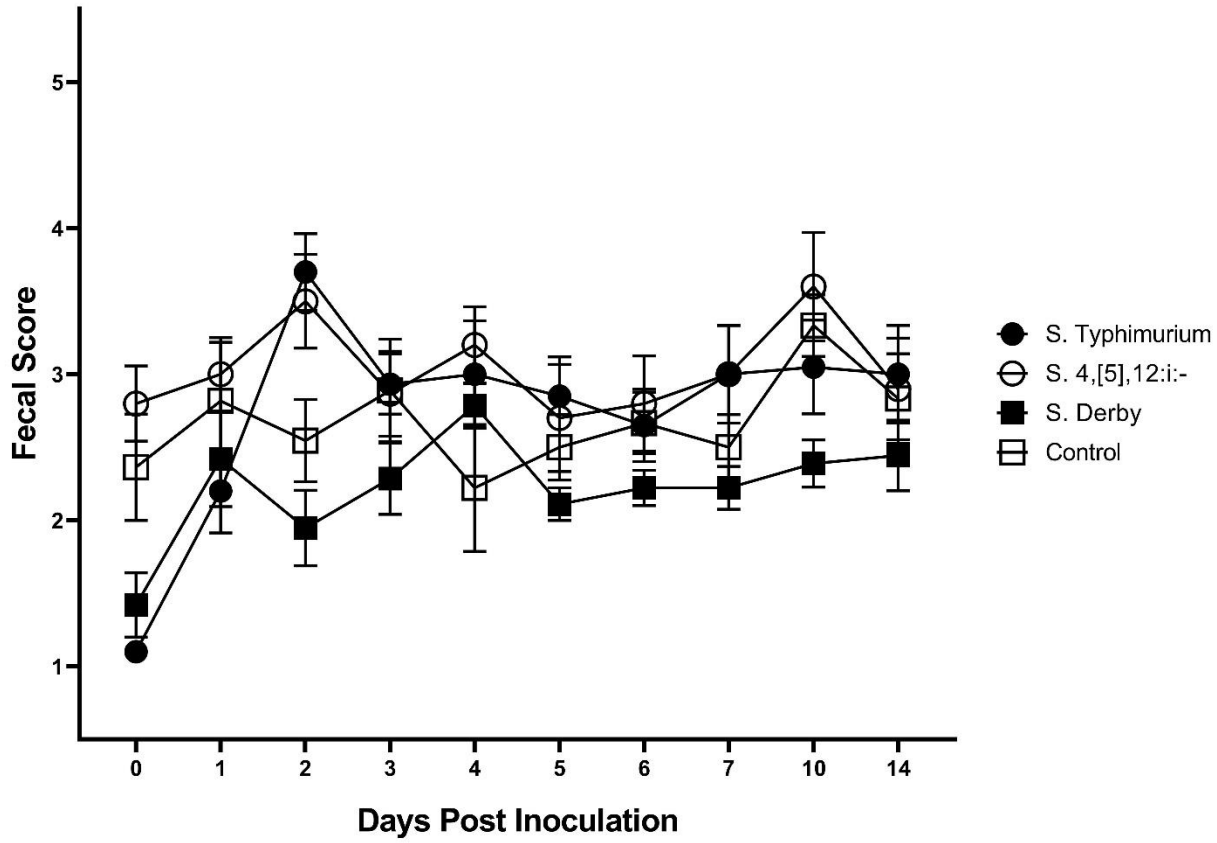


Figure 6. Comparison of fecal scores of pigs inoculated with *Salmonella* serovars 4,[5],12:i:-, Typhimurium, and Derby and non-inoculated control pigs in animal study #2. Symbols represent the mean, with vertical bars representing standard error of the mean. Fecal scores of 1-2 are normal, 3 is mild diarrhea, 4 is moderate diarrhea, and 5 is severe diarrhea.

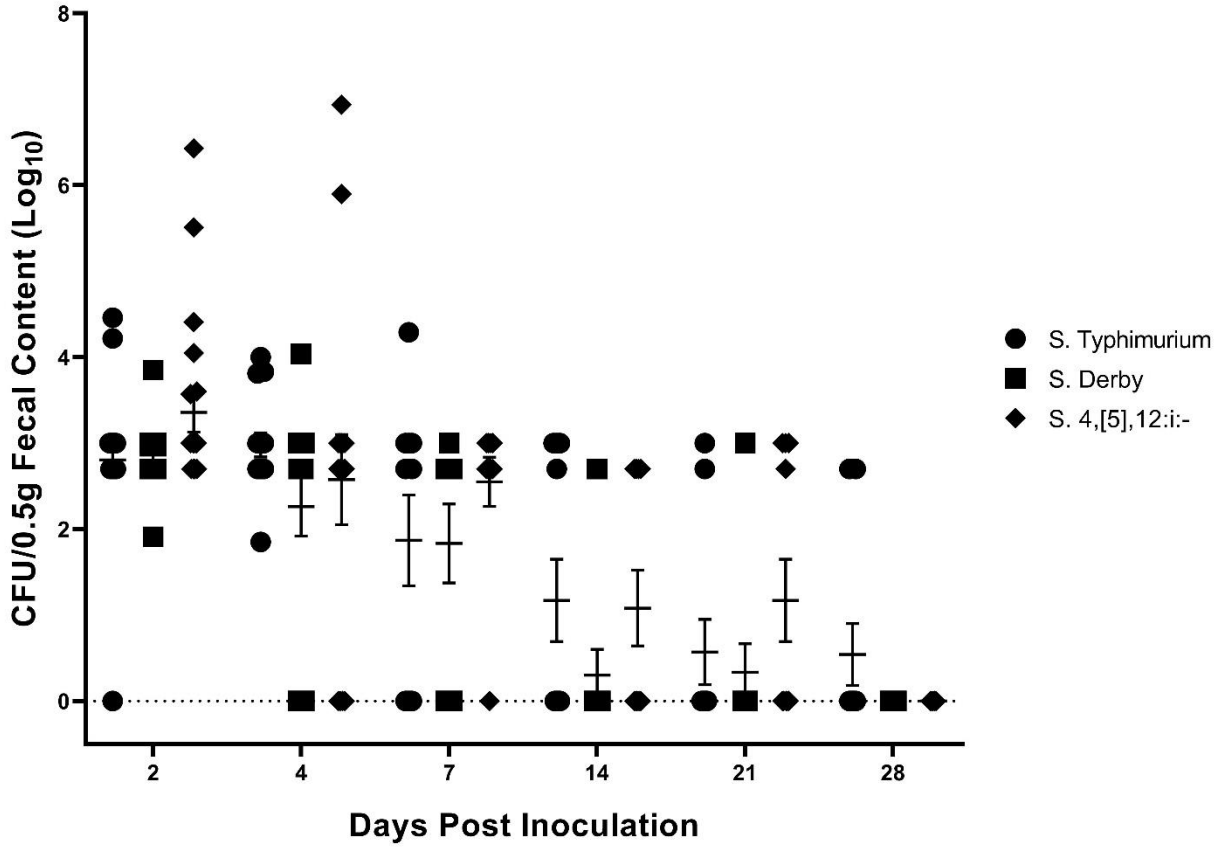


Figure 7. Comparison of quantitative culture results for *Salmonella* in feces collected from pigs inoculated with *Salmonella* serovars 4,[5],12:i:-, Typhimurium, and Derby in animal study #2. The mean and standard error are represented by the horizontal and vertical lines, respectively. Statistically significant differences ($p<0.05$) were determined within each group compared to DPI 0 at which time no *Salmonella* was detectable. Significant differences were as follows: *S.* 4,[5],12:i:- on DPI 2, 4, and 7; *S.* Derby on DPI 2, 4, and 7; *S.* Typhimurium on DPI 2, 4, 7, and 14.

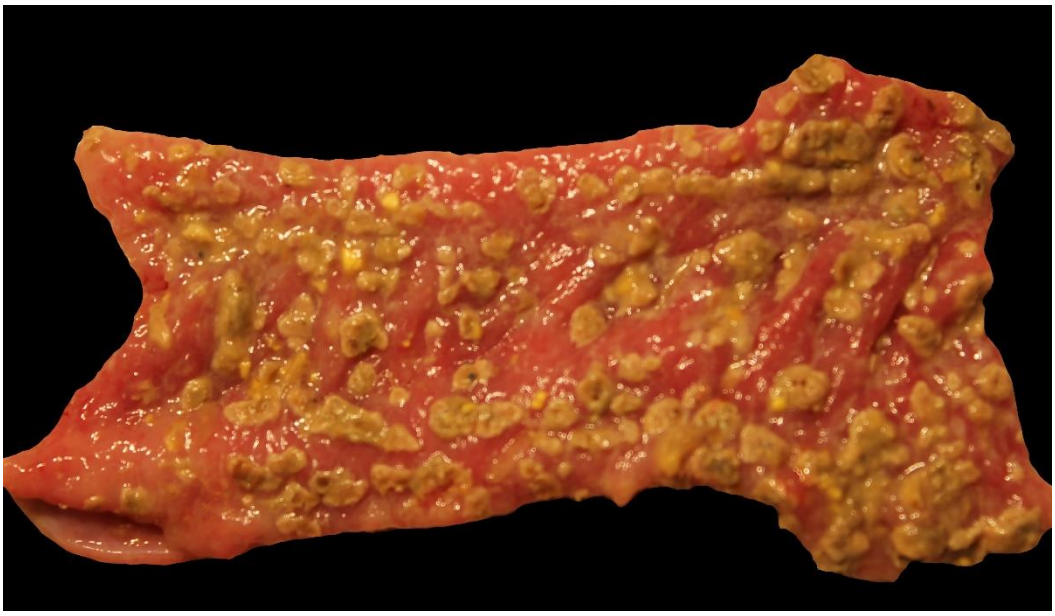


Figure 8A.

Figure 8B.

Figure 8. Representative gross lesions following infection with *Salmonella* 4,[5],12:i:- and *Salmonella* Typhimurium. Spiral colon. (7A) Pig inoculated with *Salmonella* 4,[5],12:i:-, DPI 4, severe diffuse fibrinous exudate. (7B) pig inoculated with *Salmonella* Typhimurium, DPI 4, severe diffuse fibrinous exudate.

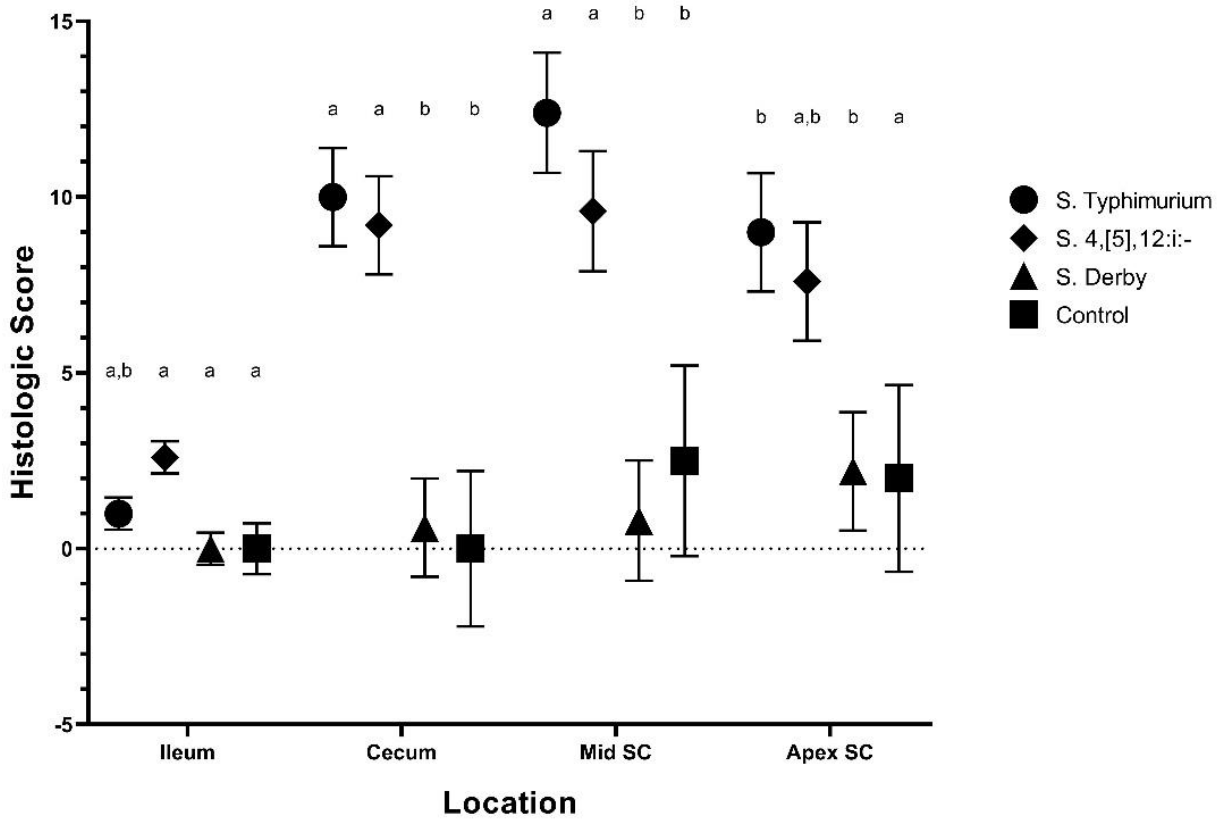


Figure 9A.

Figure 9. Comparison of histologic lesion scores from pigs inoculated with *Salmonella* serovars 4,[5],12:i:-, Typhimurium, and Derby and non-inoculated control pigs in animal study #2. Histologic lesion scores represent a summary of the ulceration, neutrophil infiltration, crypt elongation and abscessation, and submucosal inflammation at the time of necropsy on (9A) DPI 2, (9B) DPI 4, and (9C) DPI 28. Mean and standard error are represented by the symbols and vertical lines, respectively. Different letters indicate statistically significant differences between serovar groups, separated by tissue location ($p<0.05$).

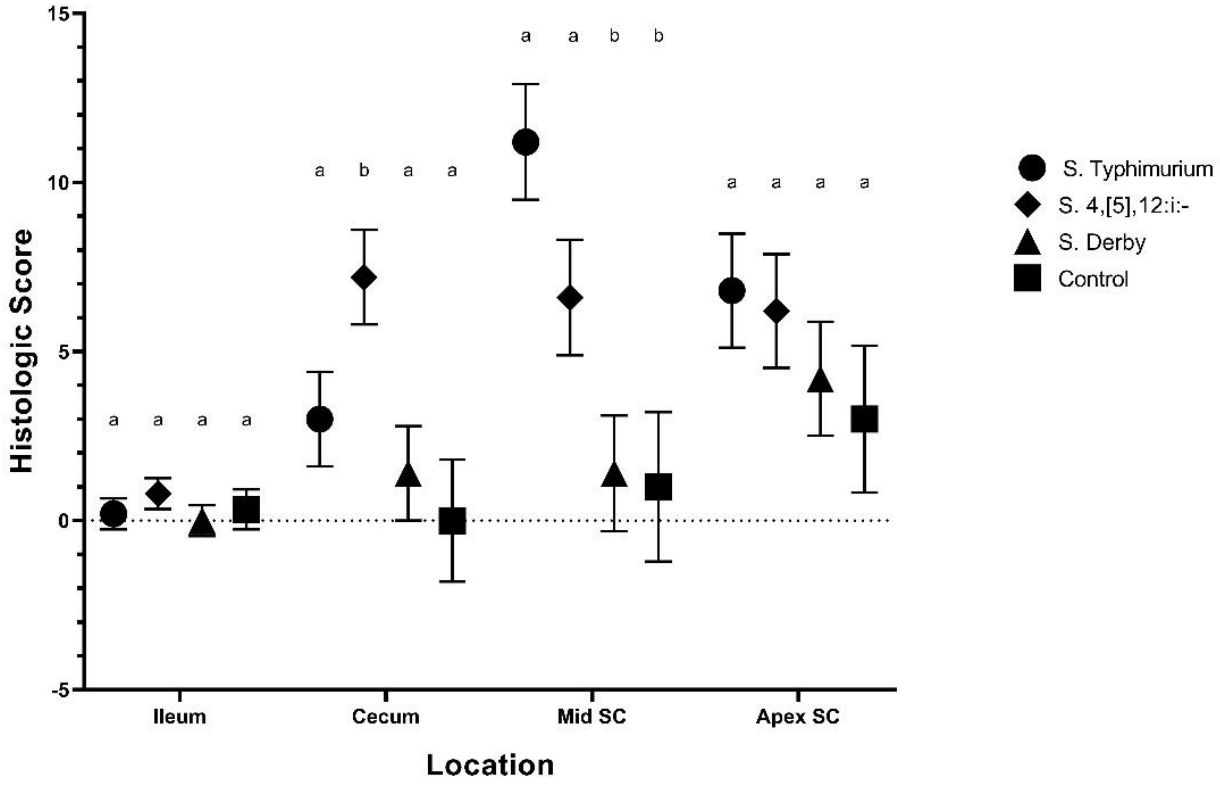


Figure 9B. (continued)

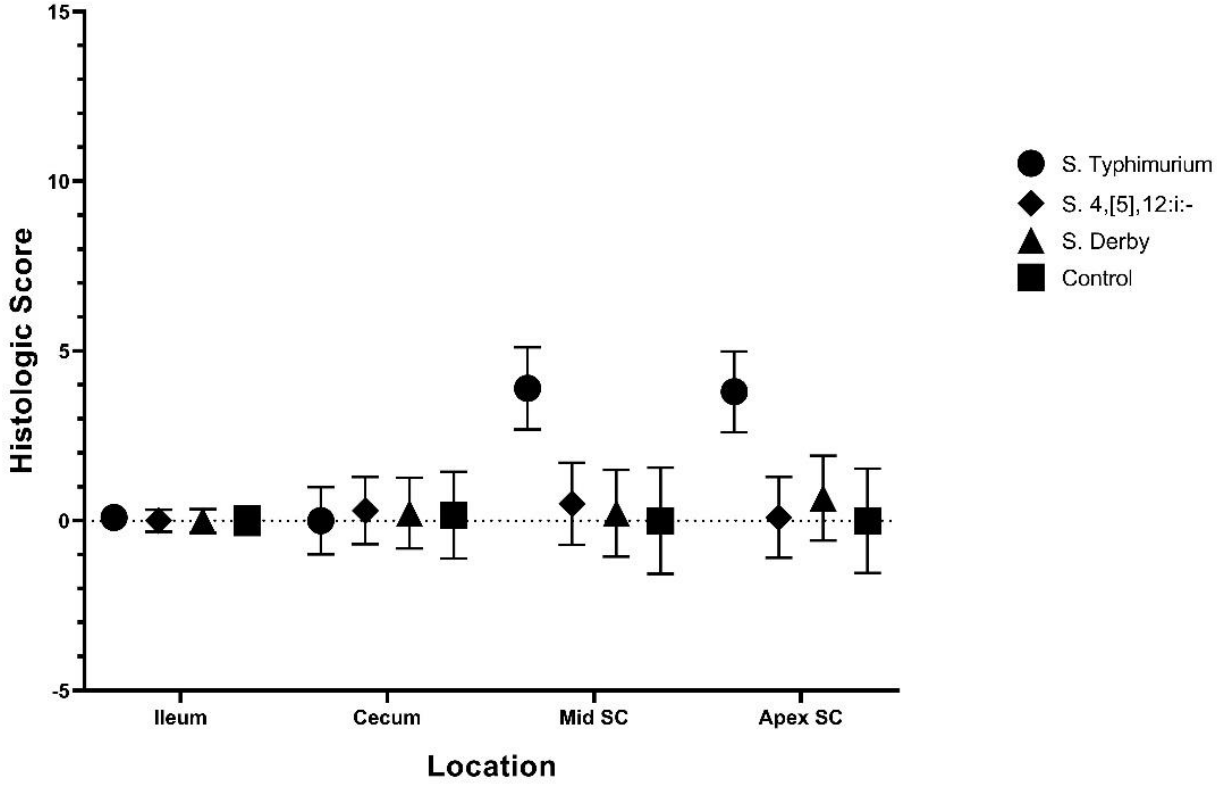


Figure 9C. (continued)

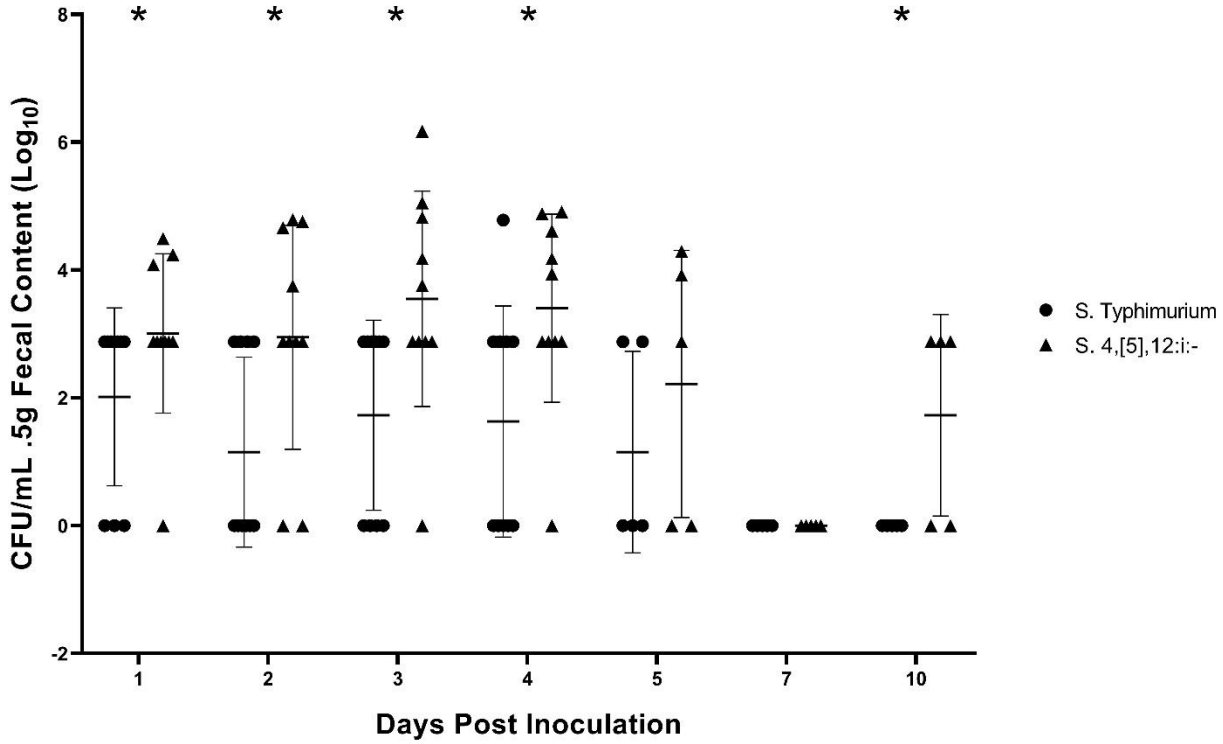


Figure 10. Quantitative fecal culture results from animal study #3 in which pigs were simultaneously co-inoculated with equal amounts of two serovars of *Salmonella*: 4,[5],12:i:- and Typhimurium. The mean and standard error are represented by the horizontal and vertical lines, respectively. Asterisks represent days in which the mean amount of *S.* 4,[5],12:i:- was significantly different from the mean amount of *S.* Typhimurium ($p<0.05$).

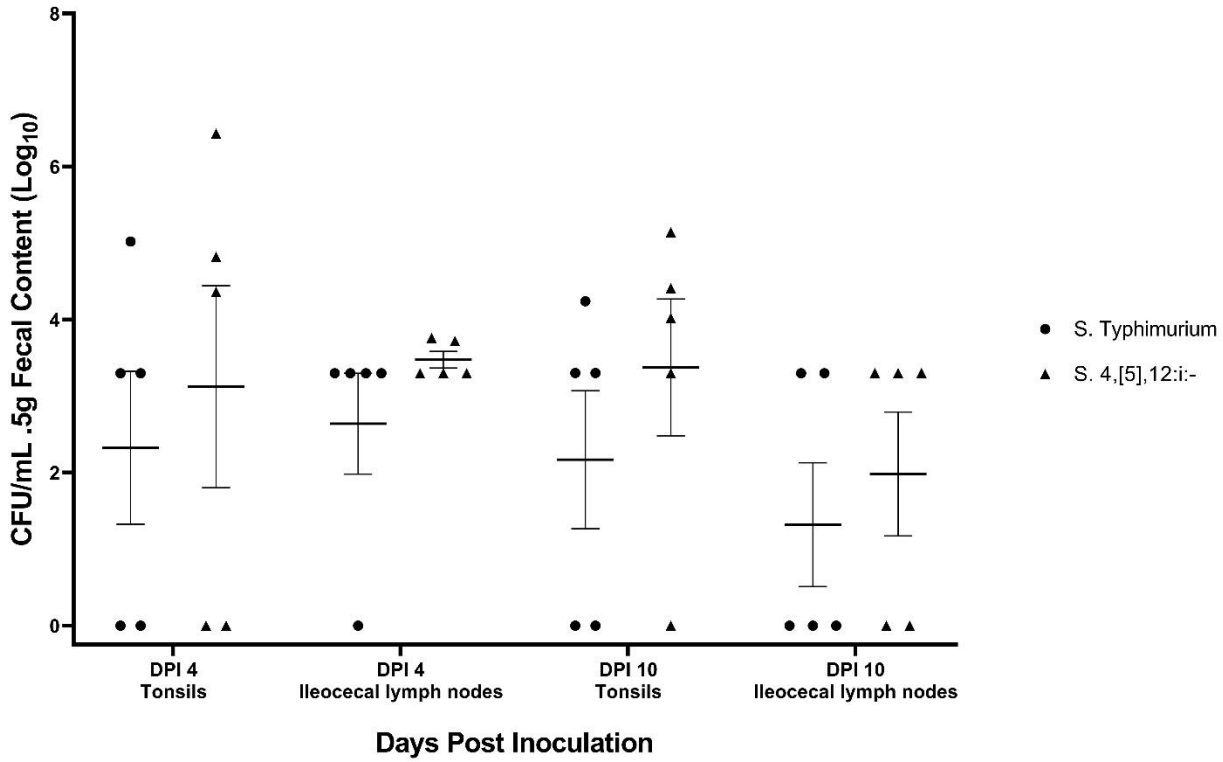


Figure 11. Quantitative culture results from samples collected upon necropsy in animal study #3 in which pigs were simultaneously co-inoculated with equal amounts of two serovars of *Salmonella*: 4,[5],12:i:- and Typhimurium. The mean and standard error are represented by the horizontal and vertical lines, respectively.

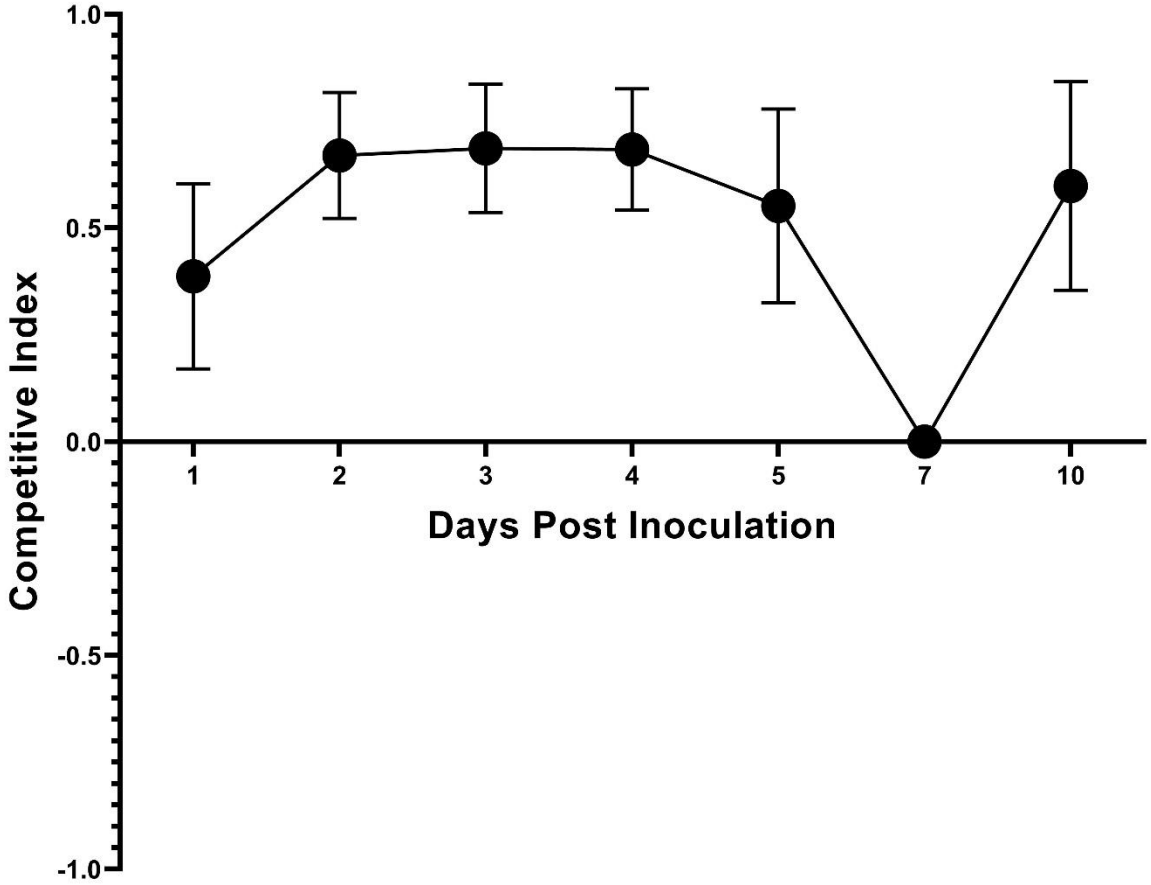

1.0
0.5
0.0
-0.5
-1.0
Competitive Index
1
2
3
4
5
7
10
Days Post Inoculation

Figure 12A.

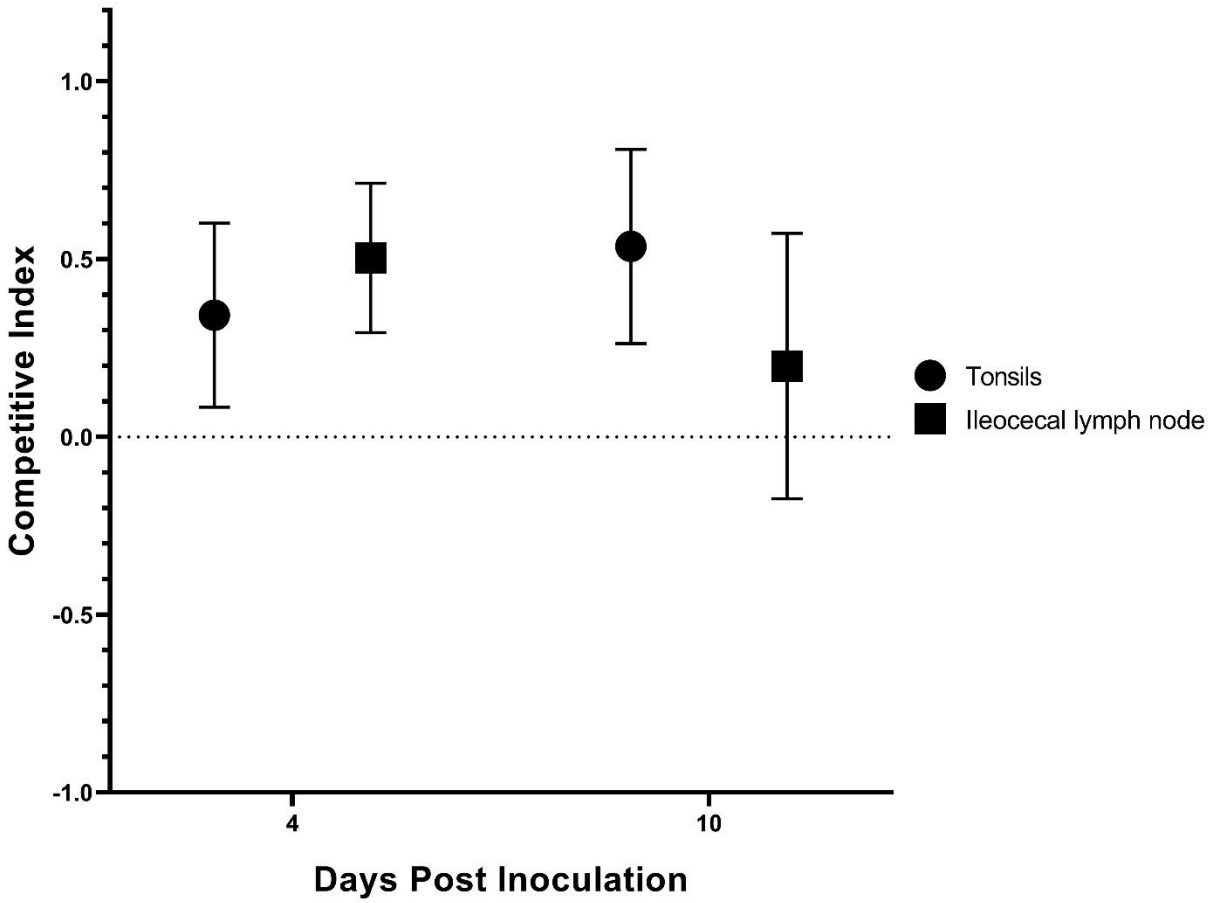


Figure 12B.

Figure 12. The competition index calculated based on the culture results from animal study #3. The competition index (CI) was calculated for (12A) fecal samples collected throughout the study period and (12B) samples collected at the time of necropsy on DPI 4 and 10. The CI represents the fitness of *Salmonella* 4,[5],12:i:- relative to *Salmonella* Typhimurium, with a positive CI indicating that *S.* 4,[5],12:i:- is more fit within the host and a negative CI indicating that *S.* Typhimurium is more fit within the host. Values closer to 1 or -1 indicates that 4,[5],12:i:- or Typhimurium were the dominant *Salmonella* serovar in that set of samples, respectively.

CHAPTER 4:

SUMMARY AND FUTURE DIRECTIONS

The objective for this thesis was to gain a better understanding of the increasing prevalence of *S.* 4,[5],12:i:-, to validate a more rapid testing method for *Salmonella*, to evaluate the disease, persistence, and colonization induced by *Salmonella* 4,[5],12:i:- within the swine host, and finally to compare the *in vivo* fitness of *S.* 4,[5],12:i:- relative to *S.* Typhimurium. *Salmonella* 4,[5],12:i:- has increased rapidly in prevalence in swine samples submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) since 2011. In conjunction with the increase in *S.* 4,[5],12:i:-, there has been a substantial decrease in prevalence of *S.* Typhimurium and other serovars within *Salmonella* serogroup B. To enable rapid detection of *Salmonella* species from swine samples, a multiplex real-time PCR was validated for the ISU-VDL. This PCR is also able to differentiate *S.* 4,[5],12:i:- and *S.* Typhimurium, likely pathogens of swine, from other serovars considered to be of lesser pathogenicity. Additionally, use of the PCR facilitates identification at the serovar-level within 1-2 days of sample submission, rather than the 3-6 weeks expected with standard culture and serotyping.

To improve the understanding of *S.* 4,[5],12:i:- as a pathogen of swine, pigs were infected with *S.* 4,[5],12:i:-, *S.* Typhimurium, or *S.* Derby to allow comparison of the recently emerging serovar to better known serovars. *Salmonella* 4,[5],12:i:- is able to induce similar levels of fever, diarrhea, and gross and histologic lesions as *S.* Typhimurium and more severe than that of *S.* Derby. Additionally, all three serovars were able to colonize tissues throughout the pig and persist for at least 28 days following infection. In an effort to better understand the cause of emergence of *S.* 4,[5],12:i:- over the past decade, pigs were simultaneously infected with equal amounts of *S.* 4,[5],12:i:- and *S.* Typhimurium. From this, it was determined that *S.* 4,[5],12:i:-

may possess a greater fitness level *in vivo* compared to *S.* Typhimurium, evidenced by higher amounts of *S.* 4,[5],12:i:- recovered from fecal and tissue samples.

Although the work completed here provides a better understanding of the monophasic variant of *S.* Typhimurium, *Salmonella* 4,[5],12:i:-, additional research is needed. During validation of the rt-PCR, the cause of unexpected results of the *fljB* gene were not able to be fully explored. Future research to understand the cause of these unexpected results as well as improved characterization of *S.* 4,[5],12:i:- isolates from swine from the Midwestern United States through whole genome sequencing or other methods of analysis may aid in understanding the cause of emergence of the monophasic variant. Additionally, only one potential mechanism to explain the emergence of *S.* 4,[5],12:i:- was explored in this study, which was the potential increased competitive fitness relative to *S.* Typhimurium within the host. This competitive fitness work should be repeated with multiple *S.* 4,[5],12:i:- and *S.* Typhimurium isolates to ensure that the competitive fitness of *S.* 4,[5],12:i:- observed remains consistent across other isolates. Many other potential explanations for its emergence are plausible and also warrant future work; these include increased survival in the environment, increased ability to be transmitted, increased antimicrobial resistance, and increased heavy metal resistance. With a better understanding of the cause of emergence and characteristics of *Salmonella* 4,[5],12:i:- infections in swine, further work is warranted on appropriate and effective control and prevention strategies. One of the necessary components should focus on currently available vaccines for *S.* Typhimurium and the potential cross protection provided by these vaccines against *S.* 4,[5],12:i:-.