

# Quantitative Microbiological Evaluation of *Salmonella* Typhimurium Shed in Diarrhea, Loose, and Normal Stools of Infected Pigs

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Received 11 March 2014; revised 6 April 2014; accepted 14 April 2014

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## Abstract

Control of within-herd transmission of *Salmonella* is important for reducing the prevalence of this organism on pig farms and for preventing *Salmonella*-contamination of pork. At the farm level, understanding the within-herd transmission of *Salmonella* can lead to more effective control. *Salmonella* infection is dependent on the inoculation dose; hence, quantitative evaluation of *Salmonella* shed in feces would provide useful information for developing effective measures. In this study, to reproduce and evaluate the number of *Salmonella* shed in diarrhea, loose stools, and normal feces, weaned pigs were inoculated with  $3.2 \times 10^9$ ,  $3.2 \times 10^7$ , and  $3.2 \times 10^5$  cfu of *Salmonella* Typhimurium, respectively. The number of *S. Typhimurium* shed in the feces peaked within 1 week post-inoculation in every group and the most amount of diarrhea and loose stools were observed within 2 weeks post-inoculation. Diarrhea occurred 10 times (six pigs), and loose stools were observed 25 times (11 pigs). The average concentration of *S. Typhimurium* shed in diarrhea, loose stools, and normal feces was  $1.0 \times 10^8$ ,  $1.6 \times 10^4$ , and  $7.1 \times 10^1$  cfu/g feces, respectively. These data suggest that diarrhea and loose stools are significant sources of within-herd transmission of *Salmonella*. Moreover, as some of the normal feces contained  $>1.0 \times 10^6$  cfu/g of *S. Typhimurium*, even normal feces could be a source of within-herd transmission of *Salmonella*. At *Salmonella*-positive farms, reduction of the amount of *Salmonella* shed even in normal feces would lead to better control of within-herd transmission of *Salmonella*. These data can contribute to the control of within-herd transmission of *Salmonella*, particularly during the weaning period.

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**How to cite this paper:** Tanaka, T., et al. (2014) Quantitative Microbiological Evaluation of *Salmonella* Typhimurium Shed in Diarrhea, Loose, and Normal Stools of Infected Pigs. *Open Journal of Veterinary Medicine*, 4, 58-66.  
<http://dx.doi.org/10.4236/ojvm.2014.44007>

## Keywords

Fecal Shedding, Quantitative Evaluation, *Salmonella* Typhimurium, Swine, Transmission

## 1. Introduction

*Salmonella* infections of swine are important because *Salmonella* can cause salmonellosis in pigs and contaminate pork products and induce human food-borne disease [1]. It has been estimated that, in the 1990s, about 20% of all human salmonellosis cases in the European Union were linked to the consumption of pork [2]. Implementing good biosecurity and operating at a high hygiene standard at the farm level itself is crucial for controlling *Salmonella* infections and for preventing human salmonellosis [3].

Controlling within-herd transmission of *Salmonella* leads to effective control of *Salmonella* infection on pig farms. To date, many studies have been performed to establish measures for controlling within-herd transmission of *Salmonella* [4]-[6]. A number of such studies attempted to mimic the situation at the herd level, in order to investigate the risk of transmission to naïve pigs when they are exposed to contaminated pens or infected pen mates [7] [8]. These studies clarified that the symptoms caused by a *Salmonella* infection and the amount of *Salmonella* shed in the feces are dependent on the inoculation dose [9]-[12]. However, most of these studies were focused on pigs in the fattening or finishing period, or pigs at abattoirs because the prevalence of *Salmonella* during these periods is directly related to *Salmonella* contamination of pork.

*Salmonella* Typhimurium is most frequently isolated serotype from pigs [13]. *S.* Typhimurium is a causative agent of pig enterocolitis and is the major agent of human food-borne disease [14] [15]. Pigs are susceptible to *S.* Typhimurium-induced diarrhea during the weaning period, and clinical salmonellosis is prevalent in these pigs at many farms in Japan and other countries [14]-[17]. Control of within-herd transmission of *Salmonella* during the weaning period reduces the prevalence of *Salmonella* during the fattening or finishing periods [18] [19]. One of the important targets for controlling the within-herd transmission of *Salmonella* during the weaning period is avoiding fecal deterioration caused by *S.* Typhimurium, as it is widely accepted that diarrhea samples contain large amounts of the organism. However, there have been insufficient scientific assessments of the amount of *S.* Typhimurium shed in diarrhea, loose stools, and normal feces.

Thus, the purpose of this study was to evaluate the amount of *S.* Typhimurium shed in diarrhea, loose stools, and normal feces after *S.* Typhimurium-inoculation of weaned pigs. These data can guide effective measures for controlling within-herd transmission of *Salmonella* during the weaning period, at the farm level.

## 2. Materials and Methods

This study was approved by the Animal Care and Use Committee of the Institute of Animal Health of JA Zen-Noh (National Federation of Agricultural Co-Operative Associations) and all animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals established by this Committee.

### 2.1. Experimental Design

Fifteen 28-day-old specific pathogen-free (SPF) pigs were transported to our isolated facilities. Upon arrival, the pigs were assigned randomly to one of three groups ( $n = 5$ ), namely, ST-9, ST-7, or ST-5; there were no weight differences among the groups. They were provided feed and water *ad libitum*.

During 7 days of acclimatization, fecal samples were collected on three occasions and serum samples were collected once to verify that the pigs were free of *Salmonella*, using culture tests and a commercial ELISA (SALMOTYPE® Pig Screen; Labor Diagnostik Leipzig, Leipzig, Germany) on the basis of previous reports [20] [21]. Based on these test results, all pigs were confirmed to be free from *Salmonella*.

The pigs were then inoculated with  $3.2 \times 10^5$  (ST-5),  $3.2 \times 10^7$  (ST-7), or  $3.2 \times 10^9$  (ST-9) cfu of rifampicin-resistant *S.* Typhimurium strain 116 (ST116Rif), respectively, to induce diarrhea and loose stools, as previously described [9]. Animals with severe salmonellosis, *i.e.*, pigs presenting with severe dehydration and depression, were euthanized by exsanguination after deep anesthesia with sodium pentobarbital (100 mg/kg), and

subjected to necropsy. At 39 DPI, surviving pigs were euthanized as discussed above and also subjected to necropsy.

## 2.2. Inoculated Culture

The *S. Typhimurium* strain, ST116Rif<sup>r</sup>, used for inoculation in this study, was prepared as described previously [20]. Briefly, the inoculated strain ST116Rif<sup>r</sup> was cultured in 200 ml of soybean-casein digest broth (Eiken Chemical Co., Ltd., Tokyo, Japan) for 5 h at 37°C, with vigorous shaking. This culture was then diluted to the appropriate concentrations using sterile phosphate-buffered saline (PBS), and then mixed with an equal volume of 20% skimmed milk. The pigs were then individually inoculated with 10 ml of this mixture via oral gavage. Specifically, each group of pigs was administered an oral dose of 10 ml containing  $3.2 \times 10^4$  (ST-5),  $3.2 \times 10^6$  (ST-7), or  $3.2 \times 10^8$  (ST-9) cfu/ml of ST116Rif<sup>r</sup>.

## 2.3. Clinical Investigations and Collection of Samples

All pigs were clinically examined on a regular basis. Clinical investigation included scoring of the condition of the feces. A fecal condition score of 0, 1, or 2 was assigned based on whether the feces appeared normal, loose, or diarrheal, respectively. Fecal samples were collected at 0, 3, 4, 6, 12, 15, 19, 22, 26, 29, 33, and 36 days post-inoculation.

## 2.4. Quantitative Culture of *S. Typhimurium* 116Rif<sup>r</sup> from Feces

After collection, 1 g of the fecal samples was diluted serially in 10-fold increments in sterile PBS and plated on deoxycholate hydrogen sulfide agar (Eiken Chemical Co. Ltd.) containing up to 100 µg/ml of rifampicin (RFDHL). The samples were then incubated for 24 h at 37°C, and the resulting colonies were counted. Next, enrichment and delayed secondary enrichment procedures were conducted. Briefly, 1 ml of a 1 g fecal solution was diluted in 10 ml of Hajna tetrathionate broth (Eiken Chemical, Co., Ltd.) followed by a 24-h incubation at 41.5°C, or for an additional 7 days at room temperature, as a delayed secondary enrichment culture. After incubation, each culture was streaked on RFDHL plates. Enrichment procedures were discontinued if positive results were obtained before the experiment was completed.

## 2.5. Qualitative Culture of *S. Typhimurium* 116Rif<sup>r</sup> at Necropsy

At necropsy, 1 g each of liver, lung, spleen, tonsil, kidney, and ileocecal lymph node tissue, as well as of the contents of the cecum and the jejunum, were aseptically collected and placed in 10 ml of Hajna tetrathionate broth. Samples were then incubated for 24 h at 41.5°C, or for an additional 7 days at room temperature as a delayed secondary enrichment culture. After incubation, each culture was streaked on RFDHL plates.

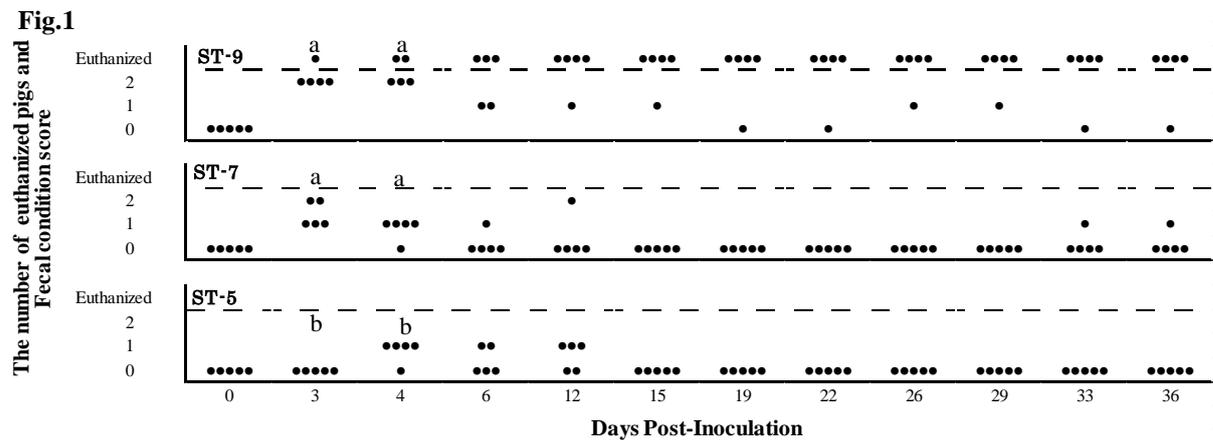
## 2.6. Statistical Analysis

The number of ST116Rif<sup>r</sup> colonies was then log transformed, with zero interpreted as 1 organism/g of feces. A value of 100 organisms/g of feces was assigned to samples that were determined to be negative by direct plating methods, but which were positive by enrichment and secondary enrichment procedures [20] [21]. The total number of ST116Rif<sup>r</sup> was expressed as means  $\pm$  standard error. Statistical differences among groups were determined using a two-sided Student's *t*-test. In addition, the fecal condition scores obtained from the three groups were compared using the Mann-Whitney *U* test. Differences with  $P < 0.05$  were considered significant.

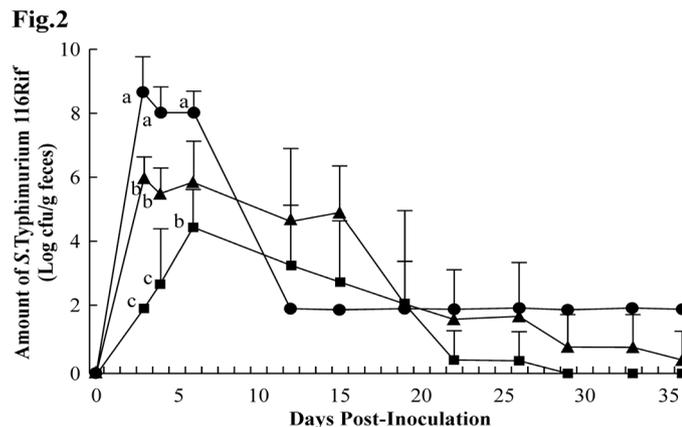
## 3. Results

### 3.1. Appearance of Salmonellosis and Fecal Conditions

The pigs were maintained in a strictly controlled environment to ensure that the diarrhea was directly caused by *S. Typhimurium* inoculation. In group ST-9, four of five pigs were euthanized because of severe salmonellosis at 3, 4, 5, and 7 DPI (Figure 1). In groups ST-5 and ST-7, all of the pigs survived until the end of the experiment. All of the pigs in group ST-9 presented with diarrhea, except for one pig, which was euthanized due to severe salmonellosis before diarrhea could be confirmed. In group ST-7, two pigs had diarrhea and all of the



**Figure 1.** Severe salmonellosis, diarrhea, and loose stools induced by *Salmonella* Typhimurium 116Rif<sup>r</sup> inoculation. Each dot represents one pig. The pigs were each inoculated orally with  $3.2 \times 10^9$  (ST-9),  $3.2 \times 10^7$  (ST-7), or  $3.2 \times 10^5$  (ST-5) cfu of *S. Typhimurium* 116Rif<sup>r</sup>. The pigs were euthanized if severe salmonellosis was observed. The condition of the feces produced by these pigs was scored. A fecal condition score of 0 indicated normal feces, 1 indicated loose stools, and 2 indicated diarrhea. Significant differences in the fecal condition score were observed between a and b ( $P < 0.05$ ).



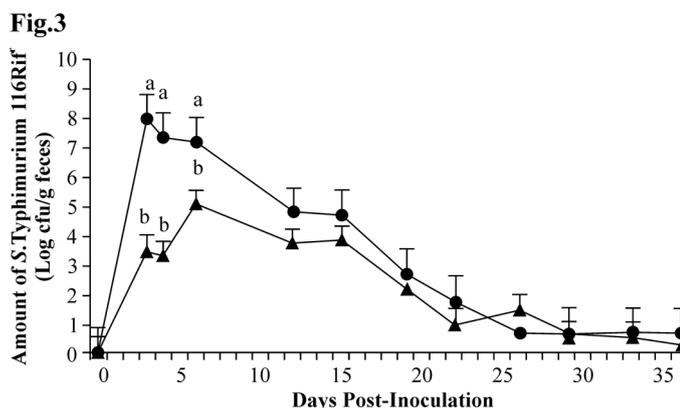
**Figure 2.** Amount of *Salmonella* Typhimurium 116Rif<sup>r</sup> shed in feces after inoculation with varying doses of *S. Typhimurium* 116Rif<sup>r</sup>. The pigs were each inoculated orally with  $3.2 \times 10^9$ ,  $3.2 \times 10^7$ , or  $3.2 \times 10^5$  cfu of *S. Typhimurium* 116Rif<sup>r</sup>, as represented by a circle, triangle, and square, respectively. Significant differences in the amount of *S. Typhimurium* 116Rif<sup>r</sup> shed in the feces were observed among the different groups (a, b, and c) ( $P < 0.05$ ).

pigs produced loose stools. In group ST-5, none of the pigs had diarrhea, but four of five pigs produced loose stools. The fecal condition scores of ST-5 differed significantly from those of both ST-7 and ST-9, at both 3 and 4 DPI ( $P < 0.05$ ). Most of the severe salmonellosis and diarrhea were observed within 7 DPI. Moreover, the fecal deterioration induced by *S. Typhimurium* infection was dependent on the inoculation dose.

### 3.2. Correlation between *S. Typhimurium* 116Rif<sup>r</sup> Shed in Feces and the Inoculation Dose

Fecal shedding of ST116Rif<sup>r</sup> peaked within 7 DPI in all groups (Figure 2), with the average peak of fecal ST116Rif<sup>r</sup> exceeding  $1.0 \times 10^8$  cfu/g in group ST-9,  $1.0 \times 10^6$  cfu/g in group ST-7, and  $3.2 \times 10^4$  cfu/g in group ST-5. The ST116Rif<sup>r</sup> count in the stools of the pigs in the three groups differed significantly at 3 and 4 DPI ( $P < 0.05$ ), and the ST116Rif<sup>r</sup> count in the feces of pigs in groups ST-5 and ST-9 differed significantly at 6 DPI ( $P < 0.05$ ).

In group ST-9, four of five pigs were euthanized by 7 DPI, while the surviving pig continuously shed  $1.0 \times 10^2$  cfu/g feces of ST116Rif<sup>r</sup>; thus, the fecal ST116Rif<sup>r</sup> count declined immediately in group ST-9. In group



**Figure 3.** Amount of *Salmonella* Typhimurium 116Rif<sup>r</sup> shed in the feces of diarrheal pigs and pigs producing loose stools, but not diarrhea, after inoculation with *S. Typhimurium* 116Rif<sup>r</sup>. The amount of *S. Typhimurium* 116Rif<sup>r</sup> shed in feces after oral inoculation with *S. Typhimurium* 116Rif<sup>r</sup>. The diarrhea group (n = 6; circles) was composed of pigs observed to produce diarrhea, while the loose stools group (n = 7; triangles) was composed of pigs observed to produce loose stools, but not diarrhea. Significant differences were observed between a and b ( $P < 0.05$ ).

ST-7, all of the pigs continuously shed ST116Rif<sup>r</sup> in their feces until 15 DPI, while in group ST-5, all of the pigs continuously shed ST116Rif<sup>r</sup> in their feces until 12 DPI. By the end of the experiment, fecal shedding of all of the pigs in group ST-7 and ST-5 had become intermittent.

### 3.3. Correlation between the Amounts of *S. Typhimurium* 116Rif<sup>r</sup> Shed in Feces by Pigs Producing Diarrhea and Those Producing Loose Stools

In this study, six pigs were observed to have diarrhea, *i.e.*, four and two pigs from ST-9 and ST-7, respectively (diarrhea group). In the diarrhea group, three of six pigs survived until the end of the experiment. Moreover, seven pigs were observed to produce loose stools, but were not observed to have diarrhea; these included three and four pigs from ST-7 and ST-5, respectively (loose stools group). In this group, all pigs survived until the end of the experiment.

The amounts of ST116Rif<sup>r</sup> shed in feces of the pigs belonging to the two groups were compared (Figure 3). In the diarrhea group, the peak count of ST116Rif<sup>r</sup> shed in the feces was approximately  $1.0 \times 10^8$  cfu/g, while in the loose stools group, this count exceeded  $1.0 \times 10^5$  cfu/g. The counts were significantly different between the two groups at 3, 4, and 7 DPI (all  $P < 0.05$ ).

In both the diarrhea and loose stools groups, the pigs shed ST116Rif<sup>r</sup> continuously until 15 DPI. In the diarrhea group, one of the three surviving pig continued to shed ST116Rif<sup>r</sup> until the end of the experiment, but the other two pigs showed intermittent shedding at 19 and 26 DPI, respectively. All of the pigs in the loose stools group switched to intermittent ST116Rif<sup>r</sup> shedding at 19 (two pigs), 22 (three pigs), 29 (one pig), and 33 (one pig) DPI.

### 3.4. Counts of *S. Typhimurium* 116Rif<sup>r</sup> Shed in Diarrhea, Loose Stools, and Normal Feces

In this trial, diarrhea was observed 10 times: seven times in ST-9 and three times in ST-5 pigs (Figure 1). Loose stools were observed 25 times: six times in ST-9, ten times in ST-7, and nine times in ST-5 pigs. Normal feces were observed 92 times. The amounts of ST116Rif<sup>r</sup> shed in diarrhea (n = 10), loose stool (n = 25), and normal stool (n = 92) samples were also compared.

The average counts of ST116Rif<sup>r</sup> shed in diarrhea, loose stools, and normal feces samples were  $1.0 \times 10^8$  cfu/g,  $1.6 \times 10^4$  cfu/g, and  $7.1 \times 10^1$  cfu/g, respectively (Table 1); these counts differed significantly ( $P < 0.01$ ). The counts of ST116Rif<sup>r</sup> shed in diarrhea ranged from  $1.7 \times 10^6$  -  $5.3 \times 10^9$  cfu/g. In loose stools, the highest ST116Rif<sup>r</sup> count was  $3.2 \times 10^8$  cfu/g. ST116Rif<sup>r</sup> was not detected in two loose stool samples, which were ob-

**Table 1.** *Salmonella* Typhimurium 116 Rif<sup>f</sup> shed in diarrhea, loose stools, and normal feces after inoculation with *S. Typhimurium* 116 Rif<sup>f</sup>.

Fecal Condition	Diarrhea	Loose stools	Normal feces
Not detected		2	40
Count of ST116Rif <sup>f</sup> shed in fecal samples (log cfu/g)			
2.0		6	26
<2.0–≤3.0			4
<3.0–≤4.0		3	7
<4.0–≤5.0		3	5
<5.0–≤6.0		5	5
<6.0–≤7.0	2**	4	5
<7.0–≤8.0	2	1	
<8.0–≤9.0	4	1	
<9.0–≤10.0	2		
Mean ± S.E.(Log cfu/g)	8.01 ± 1.08	4.20 ± 2.27	1.85 ± 2.01
Statistics*	a	b	c

\*Significant differences were observed among the groups (indicated by a, b, and c;  $P < 0.01$ ). \*\*The number of fecal samples that contained each number of ST116Rif<sup>f</sup>. After orally inoculating pigs with varying amounts of *S. Typhimurium* 116 Rif<sup>f</sup>, diarrhea, loose stools, and normal feces were observed 10, 25, and 92 times, respectively. The amounts of *S. Typhimurium* 116Rif<sup>f</sup> shed in each type of feces were analyzed.

**Table 2.** Detection rate of *Salmonella* Typhimurium 116Rif<sup>f</sup> at necropsy.

		Liver	Lung	Spleen	Tonsils	Kidney	Ileocecal lymph node	Jejunum content	Cecum content
Surviving pigs	Diarrhea	0/3	0/3	0/3	3/3	0/3	1/3	3/3	3/3
	Loose feces	1/7	1/7	1/7	6/7	2/7	3/7	5/7	3/7
	Normal feces	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
	Total	1/11	1/11	1/11	9/11	2/11	4/11	8/11	6/11
Euthanized pigs	Diarrhea	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	Unknown	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
	Total	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4

The 10 pigs that survived the infection with *S. Typhimurium* were necropsied at 39 DPI. Another four pigs were euthanized during the monitoring period, because of severe salmonellosis. At necropsy, liver, lung, spleen, tonsil, kidney, ileocecal lymph node tissue samples, and samples of the contents of the jejunum and of the cecum were aseptically collected, and subjected to *S. Typhimurium* detection.

tained at 33 and 36 DPI. In normal feces, the highest ST116Rif<sup>f</sup> count was  $6.7 \times 10^6$  cfu/g; no ST116Rif<sup>f</sup> was detected in 40 normal fecal samples.

### 3.5. Detection of *S. Typhimurium* 116Rif<sup>f</sup> at Necropsy

The rate of detection of ST116Rif<sup>f</sup> in liver, lung, spleen, kidney, and ileocecal lymph node tissues was higher in the euthanized pigs that had shown severe salmonellosis than in those of the surviving pigs who were sacrificed at the end of the experiment (Table 2). In the surviving pigs, ST116Rif<sup>f</sup> was detected more frequently in tonsil samples and in the contents of the cecum and jejunum. However, the detection rate did not differ between pigs in the diarrhea and the loose stools groups.

## 4. Discussion

The purpose of this study was to evaluate the amount of *S. Typhimurium* shed in diarrhea, loose stools, and normal fecal samples produced by *S. Typhimurium*-inoculated weaned pigs. The amount of *S. Typhimurium*

shed in diarrhea was significantly higher than that shed in loose and normal stools; the amount of *S. Typhimurium* shed in loose stools was also significantly higher than that shed in normal feces.

In this study, diarrhea, loose stools, and normal feces were produced by *S. Typhimurium*-inoculated weaned pigs, but the clinical symptoms and the amount of *S. Typhimurium* shed in the feces were dependent on the inoculation dose, as has been previously reported [9]-[12]. Our previous report, in which weaned pigs were inoculated with *S. Typhimurium* strain 116Rif<sup>r</sup>, the same strain as used in this study, showed similar results in terms of fecal deterioration and the amount of *S. Typhimurium* shed in feces in response to the inoculation dose [20].

Although some reports have evaluated the amount of *Salmonella* shed in feces, this is the first study that compared the actual count of *Salmonella* shed in feces according to the condition of the feces [22]-[24]. Previous reports involved the fattening or finishing period of pigs, during which pigs are not susceptible to diarrhea caused by *S. Typhimurium*.

Our study further supported existing data that showed that finishing pigs, naturally infected with *S. Typhimurium*, infrequently shed *Salmonella* at amounts  $>1 \times 10^6$  cfu/g in normal feces [25]. Some of the normal feces in our study contained *Salmonella* at concentrations of  $>1 \times 10^6$  cfu/g feces. These data suggest that during both weaning and fattening, pigs sometimes shed sufficient amounts of *Salmonella* to establish within-herd transmission, even when they produce normal feces.

Our study also showed that diarrheal stools contain high amounts of *Salmonella*; however, Casey *et al.* reported that diarrhea samples contained approximately  $10^3$  cfu/g feces in experimentally infected weaned pigs [23]. The difference between our findings and those of Casey *et al.* may be due to variability in the pathogenicity of the strains of *Salmonella* used to establish infection. A previous study showed that *Salmonella* strains vary in their pathogenicity [26].

There have been some reports about the amount of *Salmonella* required to establish within-herd transmission. A previous study had shown that  $5.0 \times 10^2$  cfu of *S. Typhimurium*/g feces was sufficient to induce within-herd transmission [27]. Moreover, Boughton *et al.* reported that exposure of pigs to a contaminated environment containing  $4 \times 10^2$  cfu of *S. Typhimurium*/100 cm<sup>2</sup> resulted in infection [28]. Additionally, pigs can become infected with *S. Typhimurium* when they are exposed to the organism at a concentration of  $10^3$  cfu/g feces [29]. These findings suggest that diarrheal and loose stools induced by *S. Typhimurium* contain significant amounts of the organism and are significant sources of *S. Typhimurium* infection at pig farms. Furthermore, in this study, some of the normal feces contained *S. Typhimurium* in excess of  $1 \times 10^6$  cfu/g feces, so that even normal feces could be a source of within-herd infection.

The pigs seen to produce diarrhea shed *S. Typhimurium* for longer periods than did pigs producing loose feces. The rate of detection of *S. Typhimurium* in the internal organs and contents of the digestive tract of pigs that had severe diarrhea was also higher than that in survived pigs observed to produce loose stools, but not diarrhea. Thus, pigs producing diarrhea or showing deterioration in fecal condition pose a high risk of becoming a source of within-herd *Salmonella* transmission, even when they have themselves recovered from diarrhea.

The hygienic measures taken to reduce the level of environmental contamination on pig farms constitute the basis for the control of *Salmonella* infection. This study revealed that diarrhea and loose stools are a significant source of *Salmonella* and pose a risk for within-herd transmission and moreover that some of the normal fecal samples contain sufficient amounts of *Salmonella* to establish within-herd transmission of *Salmonella*.

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