



Bacteriological, serological, and histopathological study of post-weaning piglets experimentally infected with *Salmonella* Typhimurium

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Abstract

In the last decades, infections by several serovars of *Salmonella* have become relevant worldwide, not only due to their impact on veterinary health and animal production, but also because they may serve as a source of infection for the human population. Pigs can be infected by several *Salmonella* serovars, being *S. Typhimurium* one of the most frequently isolated worldwide. In this context, the evaluation of strategies to control *Salmonella* in farms demands the understanding of the pathogenesis of the microorganism. Thus, the objective of this work was to carry out an experimental infection in 50-day-old pigs using a field strain of *S. Typhimurium* to evaluate the excretion of the microorganism, organs colonization and the development of histological injuries over the 13-day trial period. Disease was successfully reproduced in eight animals, and two clinical patterns were identified. In four animals, a primarily enteric pattern was observed, where the infection was limited to the gastrointestinal tract, while in another four pigs, enteric and systemic infection with variable fecal excretion and invasion of internal organs was observed. These two infectious patterns were accompanied by changes in fecal consistency and reduced daily weight gain or weight loss in the animals. The observation of two different infectious patterns highlights the need to consider the complexity of the pathogenicity of *Salmonella* Typhimurium infection in pigs.

Key words: *Salmonella* Typhimurium, Pigs, Experimental infection, Colonization, Enteric infection, Systemic infection, Bacteriological analysis, Serological analysis, Histopathological analysis

Estudio bacteriológico, serológico e histopatológico de lechones destetados infectados experimentalmente con *Salmonella* Typhimurium

Resumen. En las últimas décadas las infecciones por diferentes serovariedades de *Salmonella* han adquirido importancia a nivel mundial, no solo por su impacto en la sanidad y en la producción de los animales, sino también porque estos constituyen una fuente de infección para los humanos. Los cerdos pueden estar infectados o ser portadores de distintas serovariedades con potencial zoonótico, siendo *S. Typhimurium* una de las más frecuentemente aisladas a nivel mundial. En este contexto, la evaluación de estrategias para el control de *Salmonella* en granjas exige el conocimiento de la patogenia del microorganismo. El objetivo de este trabajo fue realizar una infección experimental en cerdos de 50 días de edad utilizando una cepa de campo de *S. Typhimurium* para evaluar la excreción del microorganismo, la colonización de órganos y el desarrollo de lesiones histológicas durante los 13 días del ensayo. Se logró reproducir la enfermedad en ocho animales y se pudo identificar dos cuadros clínicos. En cuatro animales se observó un cuadro principalmente entérico, en el que la infección estuvo limitada al tracto gastrointestinal; mientras que en otros cuatro cerdos se observó infección entérica y sistémica con excreción fecal variable e invasión de órganos internos. Estos dos cuadros infecciosos estuvieron acompañados de cambios en la consistencia de la materia fecal y menor ganancia diaria de peso o adelgazamiento de los animales. La observación de dos cuadros infecciosos diferentes muestra la necesidad de considerar la complejidad de la patogenia de la infección por *Salmonella* Typhimurium en cerdos.

Palabras clave: *Salmonella* Typhimurium, Cerdos, Infección experimental, Colonización, Infección entérica, Infección sistémica, Análisis bacteriológico, Análisis serológico, Análisis histopatológico

INTRODUCTION

Salmonella infections are a worldwide major public health concern. Non-typhoidal *Salmonella* is estimated to cause 93.8 million cases of acute gastroenteritis and 155,000 deaths each year worldwide (Rincón-Gamboa et al. 2021). According to the annual zoonosis report provided by the European Food Safety Authority (EFSA) in conjunction with the European Center for Disease Prevention and Control (ECDC), in 2021 salmonellosis represented the second zoonotic disease in the European Union (EU), causing 60,050 human cases and 71 deaths (European Food Safety Authority and European Centre for Disease Prevention and Control 2022).

This disease, usually associated with intensive animal production, mainly poultry and swine, may cause significant economic losses due to animal mortality, morbidity, and stunted growth, increased feed conversion rates and increased costs of non-specific treatments. In addition, some animals may be infected without manifesting any clinical signs of the disease and are thus relevant in the dissemination of the pathogen within farms and usually serve as sources of food contamination (Rincón-Gamboa et al. 2021). Pigs may be infected or carry a large number of serovars with zoonotic potential, including *S. enterica* serovar Choleraesuis, which causes human typhoid-like disease (Jajere 2019), and *S. Typhimurium*, which is the serovar most commonly isolated from swine worldwide, as well as humans (EFSA and ECDC 2022). This paratyphoid serovar has the ability to translocate from gut to different internal organs (Hofacre et al. 2021). Infections caused by invasive serovars are often life-threatening and require adequate and effective antibiotic therapy. However, the emergence of strains resistant to multiple antibiotics impacts on the efficacy of treatment and is considered a growing public health concern (Nair et al. 2018). Thus, it is necessary to have infection models that allow us to understand and evaluate the pathogenesis of this microorganism and that can be used in the future to test new treatments and control strategies.

The pathogenesis of *Salmonella* has been studied through both *in vitro* and *in vivo* models. Although the use of *in vitro* experimental models has generated valuable information, these do not allow understanding the complexity of the biological processes involved in the interaction of *Salmonella* with the host. Therefore, to better understand the pathogenesis of the disease, it is important to develop *in vivo* test models (Boyle et al. 2007). However, when designing an *in vivo* model, it is necessary to take into account the 3R principles, i.e., replacing *in vivo* models when possible, refining techniques and methodologies to obtain more reliable results, and reducing the number of animals for *in vivo* work.

Based on the above, the aim of the present work was to carry out an experimental infection in 50-day-old pigs using a field strain of *Salmonella Typhimurium* to evaluate the excretion of the microorganism, the colonization of organs and the development of histological injuries during the 13 days of the trial.

MATERIALS AND METHODS

Animals and maintenance conditions. Eight 50-day-old cross-breed pigs (5 males and 3 females) were obtained from an intensive production farm located in the Province of Buenos Aires, Argentina. The farm was free of Aujeszky's disease virus and *Brucella suis* and reported no clinical signs or laboratory diagnosis of ileitis or swine dysentery. At 21 days old, pigs were vaccinated with a commercial vaccine against *Mycoplasma hyopneumoniae* and Porcine Circovirus Type 2. From the day of arrival at our institution until the end of the study, the animals were maintained in Biosecurity Level 2 facilities of the Centro de Investigación en Ciencias Veterinarias y Agronómicas of the Instituto Nacional de Tecnología Agropecuaria (CICVyA, INTA), Buenos Aires (Argentina) under a natural day-night rhythm and a constant temperature of 24°C. Throughout the study, pigs received water and commercial feed *ad libitum* without antibiotics. As environmental enrichment, a container with hoses and empty plastic bottles were available to the animals. Prior to the experimental infection, pigs were confirmed to be free of *Salmonella* by bacteriological analysis of rectal swabs and did not present antibodies against *Salmonella* by indirect ELISA. The study followed the guidelines of animal welfare of INTA and the procedures were approved by the Institutional Committee for the Care and Use of Experimental Animals of the CICVyA (CICUAE #47/2017).

***Salmonella Typhimurium* strain.** Pigs were challenged with the *S. Typhimurium* INTA 2351/17-MJ strain. This strain had been previously isolated from stool samples of clinically affected fattening pigs from an intensive farm in Argentina. The strain had been previously characterized by Pulsed Field Gel Electrophoresis and virulotyping, and its susceptibility to several antimicrobials had been determined (Joaquim et al. 2021). The isolate showed multidrug resistance (multiple Antimicrobial Resistance Index of 0.63) and presented nine out of the ten virulence genes analyzed (Joaquim et al. 2021). In order to increase its virulence, the strain was previously used to infect two 70-day-old pigs and re-isolated from the mesenteric lymph nodes 5 days after infection. The strain was kept at -70°C, and to elaborate the infectious inoculum, it was cultured in Brain Heart Infusion broth (Merck, Darmstadt, Germany) at 37°C for 6 h. Thereafter, bacterial enumeration was performed using xylose-lysine-deoxycholate agar (Merck, Darmstadt, Germany) with the addition of 4.6% of tergitol 4 (SIGMA®, USA) (XLDT) (Miles et al. 1938). The suspension was kept overnight at 4°C and, according to the results, it was properly diluted to render an infective dose of 1×10^9 colony-forming units (cfu) per mL (Audisio and Terzolo 2002).

Experimental design, observations and sampling. Pigs were infected by individual oral gavage, using a plastic syringe with 3×10^9 cfu of the challenge strain contained in 3 mL of PBS pH 7.4. The infection dose was confirmed by bacterial enumeration after the inoculation.

Clinical variables such as anorexia, lethargy, reaction to external stimuli, posture and respiratory pattern, and

rectal temperature, as well as the weight of all the animals, were daily recorded and weight gain was individually calculated. Feces of each animal were observed immediately after deposition and their consistency was classified according to Pedersen et al. (2011) and assigned a representative score value: 1 (firm), 2 (pasty), 3 (creamy), or 4 (aqueous). To determine the re-isolation rate of the microorganism, individual rectal swabs were daily taken. At 13 days post-infection (DPI), pigs were euthanized through intramuscular injection with an overdose of ketamine and midazolam followed by exsanguination according to the American Veterinary Medical Association's Manual for euthanasia (Underwood et al. 2013). Blood samples were taken to determine the presence of serum antibodies against the pathogen. All animals were necropsied and the presence of macroscopic lesions in the organs from the thoracic and abdominal cavities was recorded. Samples of tonsils, thymus, lung, heart, liver, spleen, kidney, mesenteric lymph nodes, duodenum, jejunum, ileum, cecum and colon were taken for the detection of *Salmonella* and for histopathological analysis. In addition, a portion of the ileum from each pig was taken to determine the levels of specific mucosal IgA.

Detection of *Salmonella*. Tissue samples and rectal swabs were pre-enriched in 1% peptone water and incubated at 37°C for 18 h. Thereafter, 1 mL of the grown broth was sub-cultured in tetrathionate broth plus 2% lugol and 1% brilliant green at 37°C for 48 h and then sub-cultured onto XLDT agar at 37°C for 24 h (International Standard ISO 6579:2002). The presence of *Salmonella*-like colonies was observed, and their identity was confirmed by plate agglutination using polyvalent Group B *Salmonella* antisera. Also, total DNA was extracted from 1 mL of the grown tetrathionate broth and used to amplify the *Salmonella invA* gene by PCR, according to Malorny et al. (2003).

Histopathological analysis. For histological analysis, samples were immediately fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 3 µm and stained with hematoxylin/eosin (Olivieri et al. 2018). Slides were observed under the microscope at 10, 20 and 40X and microscopic lesions were recorded.

Presence of antibodies against *Salmonella*. The levels of IgG and IgA against *Salmonella* were analyzed by ELISA. The pigs were tested for antibodies in serum before the infection with *Salmonella* and the final of the trial. Polisorp microtiter plates (Thermo Scientific) were coated with 4 µg/well of purified *S. Typhimurium* lipopolysaccharide and incubated at 4°C for 18 h (SIGMA®, USA). Then, sera were diluted 1:80 with PBS plus 0.05% Tween 20 (PBS-T) and added to the plates. After incubation at 37°C for 1 h, plates were incubated with anti-pig IgG and IgA conjugated with horseradish peroxidase (HRP) (diluted 1:3000) (SIGMA®, USA). Finally, plates were revealed using the ABTS system and absorbance at 405 nm was measured (Bagul et al. 2018). Also, an agglutination test for the detection of antibodies against *Salmonella* was performed in 96-well round-bottom microtiter plates (Bustos et al. 2021). Briefly, 50 µL of two-fold serially

diluted sera (from 1:4 to 1:2048) was mixed with 50 µL of a suspension of a formalin-inactivated *S. Typhimurium* INTA 2351/17-MJ strain (circa 10⁸ cells mL⁻¹) and then incubated overnight at 37°C. Microagglutination titer was defined as the reciprocal of the highest positive dilution.

Determination of IgA titer in the mucosa of the ileum. The sample of the ileum extracted during necropsy was sectioned longitudinally and the coarse content was gently removed with the back of the finger to expose the clean mucosa and washed with PBS. Thereafter, the mucosa was scraped with a clean and disinfected slide, at an angle of 45°, and collected in a tube. Then, 500 mg of the mucosa scraping was re-suspended with 500 µL of PBS-T and 1% bovine serum albumin. The mix was centrifuged at 5000 g for 10 minutes, the pellet was discarded, and the supernatant was kept (Okamoto et al. 2007). Samples were diluted 1:80 and the levels of IgA were analyzed by ELISA using anti-pig IgA conjugated with HRP (diluted 1:5000), as previously described.

Statistical analysis. Variations in animal weight gain throughout the study were analyzed by repeated-measures analysis of variance (ANOVA) and Tukey's multiple comparison test. Differences and concordance between the detection of the microorganism by bacteriological isolation and PCR in stool or tissue samples were analyzed by the Fisher's contingency test and the Cohen's kappa coefficient respectively. All analyses were performed using the GraphPad Prism 5.0 software, with a significance level of 0.05.

RESULTS

Clinical condition. From 1 to 5 DPI, the average daily weight gain of the pigs ranged from 0.350 ± 0.2 kg to 0.575 ± 0.462 kg, whereas, from 6 DPI onwards, the animals lost weight at least one of the days of the study (Figure 1). From 1 to 3 DPI, increased rectal temperature was detected in 3 out of 8 of the pigs, with temperatures higher than 39.5°C, while, during the subsequent days, no animal showed abnormal rectal temperature values (Figure 2). From 1 to 4 DPI, the average stool score was 1 (firm), whereas, from 5 DPI onwards, the average scores increased, reaching a maximum of 2.5 (consistency between pasty and creamy) at 8 and 9 DPI. Then, from 10 DPI and until the end of the study, average scores decreased to 2 (pasty consistency) (Figure 3). Considered individually, 75% (6 out of 8) of the pigs presented firm stool at the beginning of the study and, from 6 DPI onwards, almost all pigs (6 out of 8) showed stool scores greater than 2. In general, no other abnormal clinical signs were observed. Only one pig presented depression between the 3 and 7 DPI. This pig was lethargic and laid in sternal decubitus with the limbs below the body or in lateral decubitus. However, the response to external stimuli and the respiratory pattern were not affected.

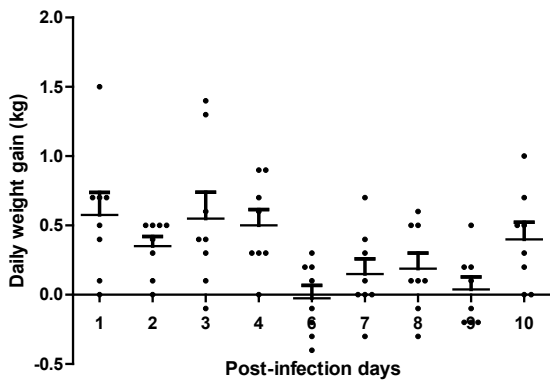


Figure 1. Average daily weight gain in 8 pigs experimentally infected with the *S. Typhimurium* INTA 2351/17-MJ strain.

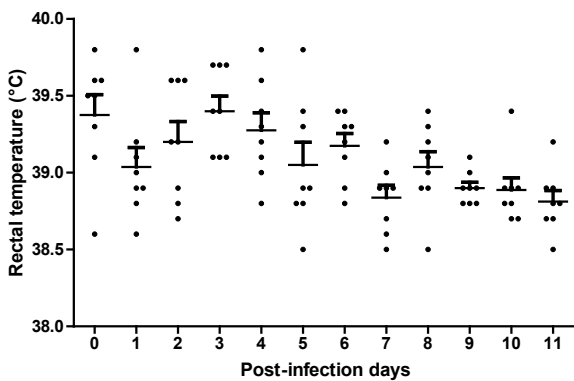


Figure 2. Average daily rectal temperature in 8 pigs experimentally infected with the *S. Typhimurium* INTA 2351/17-MJ strain.

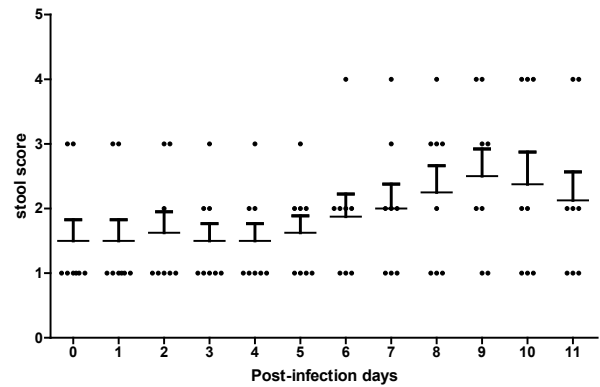


Figure 3. Average daily stool score in 8 pigs experimentally infected with the *S. Typhimurium* INTA 2351/17-MJ strain.

Shedding of *Salmonella*. The detection of *Salmonella* in the feces by bacteriological isolation and PCR is shown in Table 1. The maximum percentage of detection (50%) of the pathogen was recorded at 9 DPI. In only 4 out of the 192 samples analyzed, bacteriological isolation did not correspond to the molecular detection of the pathogen, but this difference was not significant ($p=0.5943$). The coefficient of agreement between the techniques was 0.859, with a substantial degree of agreement.

Table 1. Detection of *Salmonella* in feces by bacteriological isolation and PCR.

Pig		Days Post-infection											
		1	2	3	4	5	6	7	8	9	10	11	12
1	Bacteriological isolation	-	-	-	-	-	-	-	-	-	-	-	-
	PCR	-	-	-	-	-	-	-	-	-	-	-	-
2	Bacteriological isolation	+	-	-	-	-	-	-	+	+	+	-	+
	PCR	+	-	-	+	-	-	-	+	+	+	-	+
3	Bacteriological isolation	-	-	-	-	-	-	-	+	-	-	-	-
	PCR	-	-	-	-	-	-	+	+	-	-	-	-
4	Bacteriological isolation	-	-	-	-	-	-	-	-	-	-	-	-
	PCR	-	-	-	-	-	-	-	-	-	-	-	-
5	Bacteriological isolation	-	+	-	-	-	-	-	-	-	-	-	-
	PCR	-	+	+	-	-	-	-	+	-	-	-	-
6	Bacteriological isolation	-	-	-	-	-	-	-	-	-	+	+	+
	PCR	-	-	-	-	-	-	-	-	-	+	+	+
7	Bacteriological isolation	-	-	-	-	-	-	-	-	-	-	-	-
	PCR	-	-	-	-	-	-	-	-	-	-	-	-
8	Bacteriological isolation	+	+	+	+	+	+	+	+	+	+	+	+
	PCR	+	+	+	+	+	+	+	+	+	+	+	+

***Salmonella* colonization in the intestinal tract and translocation to internal organs.** The isolation of *Salmonella* in the different sections of the gastrointestinal tract and in the internal organs is detailed in Table 2. In these samples, the coefficient of concordance between the bacteriological isolation and PCR was 1. The highest rates

of microorganism isolation were observed in the distal portion of the intestine: 62.5% in the ileum, 50% in the cecum and 37.5% in the colon. Similarly, the pathogen was detected in 50% of the samples of mesenteric lymph nodes. Regarding internal organs, the highest isolation rates were found in the heart muscle, thymus and tonsils

(37.5%) followed by the liver, kidney and lung (25%); and the bacterium was isolated from the spleen in only one pig (12.5%). In 50% of the animals, *Salmonella* was isolated in at least one of the internal organs, while in the other 50%, it was only isolated in intestinal contents or mesenteric lymph nodes.

When the challenge strain was recovered, we observed that the colonies isolated from the internal organs

(tonsils, thymus, liver, spleen, kidney, heart and lung) presented irregular borders, which did not correspond to the macroscopic morphology typical of *Salmonella*. In contrast, the colonies isolated from the different sections of the intestinal contents and from the mesenteric lymph nodes presented typical *Salmonella* morphology. The virulotyping technique did not detect loss of any virulence gene in any *Salmonella* isolate obtained from the internal organs, intestinal contents, or mesenteric lymph nodes.

Table 2. Detection of *Salmonella* in the gastrointestinal tract and internal organs by bacteriological isolation and PCR.

Gastrointestinal tract and internal organs	Pig							
	1	2	3	4	5	6	7	8
Duodenum	+	-	-	+	-	-	-	-
Jejunum	-	-	-	+	-	-	-	+
Ileum	-	+	-	-	+	+	+	+
Cecum	-	+	-	+	-	+	-	+
Colon	-	+	-	-	-	+	-	+
Mesenteric lymph nodes	-	-	+	+	-	+	+	-
Liver	+	-	-	-	-	+	-	-
Spleen	-	-	-	-	-	+	-	-
Kidney	-	-	-	+	-	+	-	-
Heart muscle	-	-	-	+	-	+	-	+
Lung	-	-	-	+	-	-	-	+
Thymus	+	-	-	+	-	+	-	-
Tonsils	+	-	-	+	-	-	-	+

Histopathological analysis. At the time of necropsy, no macroscopic lesions were observed. In contrast, focal microscopic lesions compatible with *Salmonella* infection were observed in intestinal samples of all pigs. The lesions most frequently observed were epithelial erosion and necrosis with formation of pseudomembranes. Abscesses were observed in crypts and mononuclear exudates in the lamina propria and/or submucosa (7 out of 8 pigs) (Figure 4). Edema was mainly observed in the mesenteric lymph nodes, especially in two pigs. At a systemic level, three pigs presented focal necrosis in the liver (Figure 5), tonsils

(Figure 6) and mononuclear infiltrate in the lung (Figure 7) and in the kidney (Figure 8). The pigs with microscopic enteric lesions were the same as those from which *Salmonella* Typhimurium was isolated from intestinal contents. Similarly, pigs that presented microscopic lesions in internal organs were the same as those from which *Salmonella* was re-isolated from internal organs. We also observed a relationship between the modification of the stool score, lower daily weight gain, and the presence of lesions in the intestine.

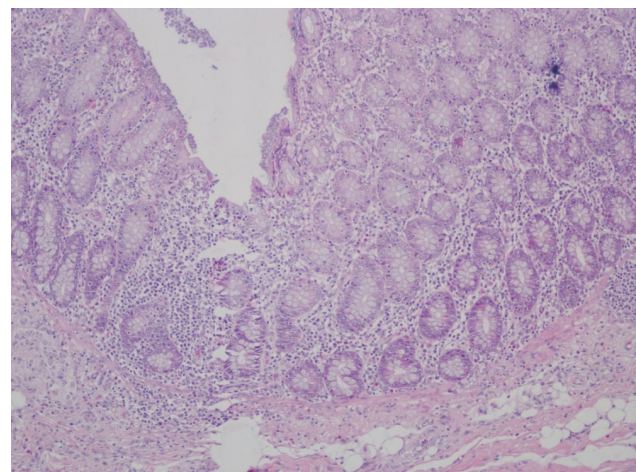
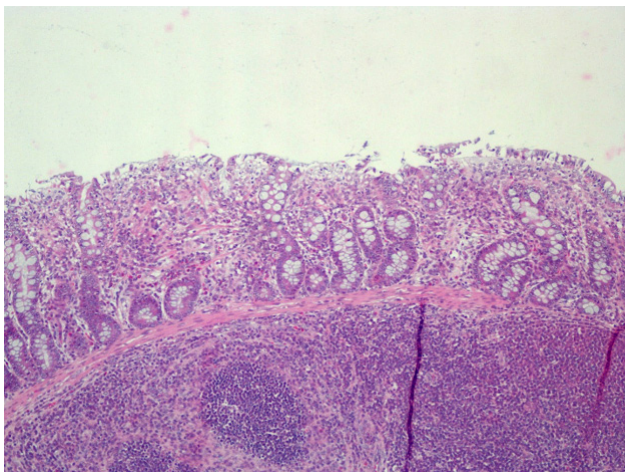


Figure 4. Histological examination of different portions of the intestine. (A) Necrosis of the mucosa of the ileum. Hematoxylin and eosin, 4x. (B) Presence of pseudomembranes and mononuclear infiltrate in the lamina propria of the colon. Hematoxylin and eosin, 10x.

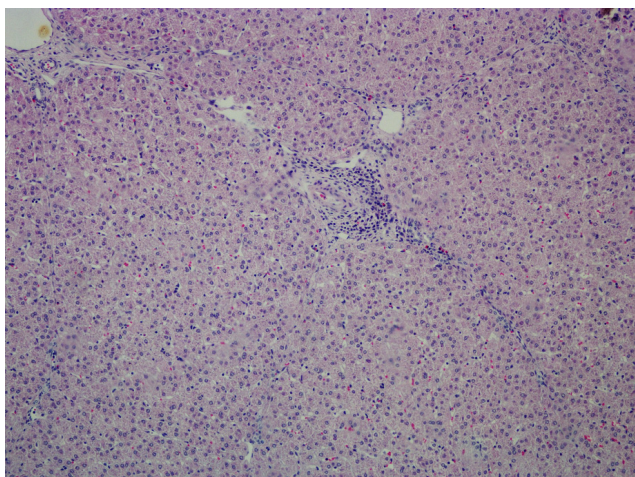


Figure 5. Foci of coagulative necrosis in the liver. Hematoxylin and eosin, 20x.

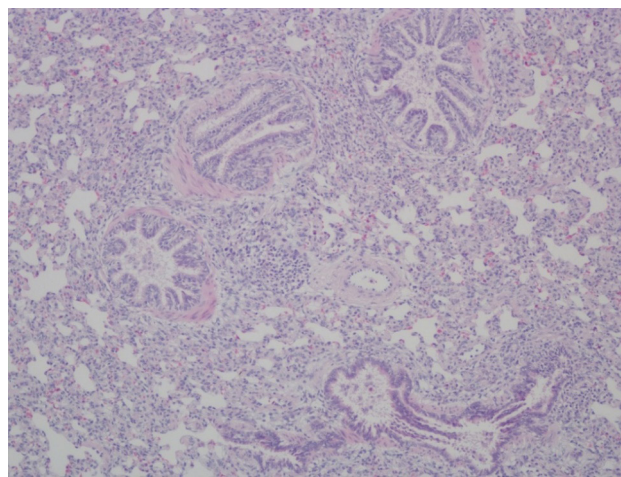


Figure 7. Thickening of the interlobular septa of the lung with the presence of mononuclear cells. Hematoxylin and eosin, 20x.

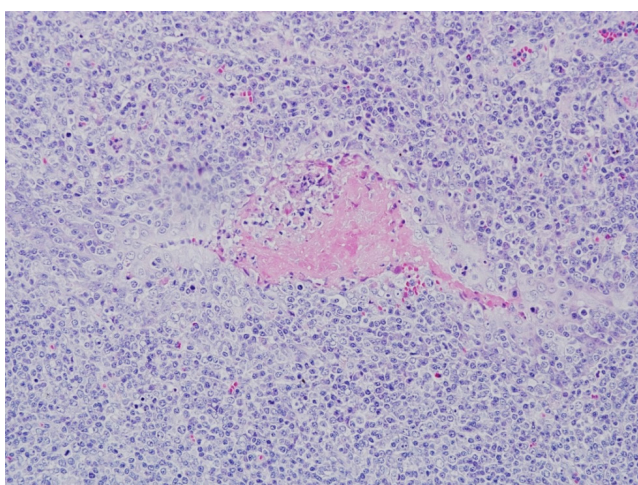


Figure 6. Foci of necrosis in the tonsillar crypt. Hematoxylin and eosin, 10x.

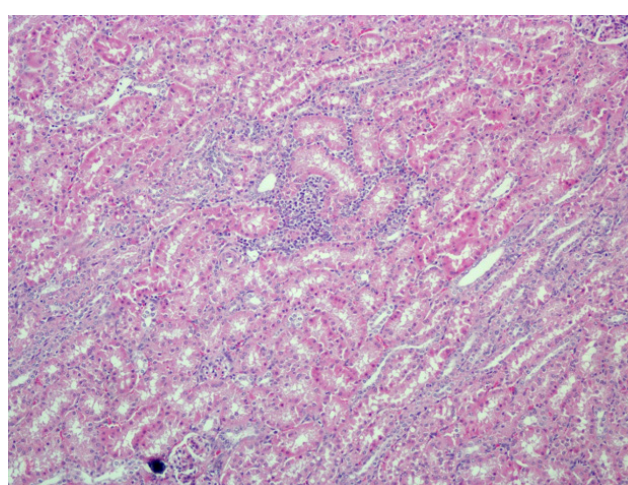


Figure 8. Mononuclear infiltrate in the renal interstitium. Hematoxylin and eosin, 10x.

Antibody responses to *Salmonella*. At the end of the trial, using the slow plate agglutination technique, all pigs showed antibodies against *S. Typhimurium* in serum, with titers ranging between 1:4 and 1:8, except pig No. 5, which had a titer of 1:128. The indirect ELISA showed that the optical density (OD) of IgG and IgA in serum presented

a lower dispersion than the OD of IgA in the mucosa of the ileum (Table 3). Figure 9 shows the OD values of IgG in serum, IgA in serum and IgA in the mucosa of the ileum for each of the pigs, as determined by the indirect ELISA technique against lipopolysaccharide O: 4,5,12 of *S. Typhimurium*.

Table 3. Antibody titers against *Salmonella Typhimurium* by slow plate agglutination in serum, and optical density (OD) values of IgG in serum, IgA in serum and IgA in the mucosa of the ileum at a dilution of 1:80 by the non-competitive indirect ELISA technique with the lipopolysaccharide O: 4,5,12 of *S. Typhimurium* (LPS).

Pig	Slow plate agglutination (Titer)	ELISA - LPS		
		IgG in serum (OD)	IgA in serum (OD)	IgA in the mucosa of the ileum (OD)
1	1:8	0.3594	0.6277	0.0631
2	1:4	0.2457	0.6986	0.0790
3	1:8	0.2133	0.5347	0.9740
4	1:4	0.1747	0.7652	0.2566
5	1:128	0.2159	0.4732	0.0557
6	1:8	0.1943	0.5071	0.1096
7	1:4	0.2216	0.6531	0.1821
8	1:8	0.3767	0.7035	0.0650

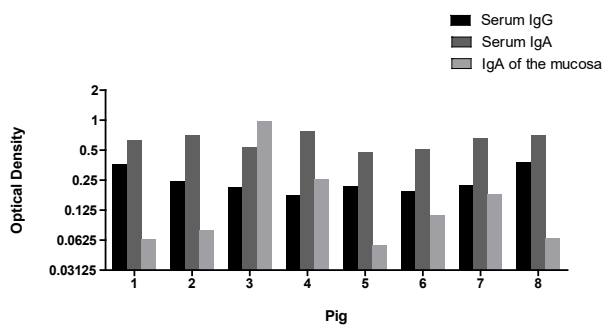


Figure 9. Optical density of IgG in serum, IgA in serum and IgA in the mucosa of the ileum at a dilution of 1:80 for each of the pigs by means of the indirect ELISA technique with the lipopolysaccharide O 4,5 of *S. Typhimurium*.

DISCUSSION

In this work, an experimental infection of pigs was carried out using a virulent strain of *Salmonella* Typhimurium to evaluate the excretion and organ colonization of the pathogen in post-weaning piglets. We decided to use this serovar because it is one of the most frequently isolated from pigs both in Argentina (Colello et al. 2018) and in the world (EFSA and ECDC 2022). In addition, *Salmonella* Typhimurium is one of the most prevalent serovars involved in foodborne outbreaks in humans (EFSA and ECDC 2022).

We were able to reproduce the infection with *S. Typhimurium* in all the animals. Throughout the study (which lasted 13 days), it was possible to evaluate the clinical signs in the tested animals, the isolation of *Salmonella* in the feces, and in the internal organs, the serological and histopathological analysis. The increase in temperature here observed at the beginning of *Salmonella* infection is in agreement with that previously described by several authors, who observed that the rectal temperature of animals increases above normal values during the first three days following infection (Boyen et al. 2009, Cevallos-Almeida et al. 2018, Naberhaus et al. 2020). This would be associated with the process of invasion of the microorganism into the bloodstream (Mandal and Brennan 1988).

We also observed changes in the consistency of the pig feces, also in agreement with that reported by other authors (Boyen et al. 2009, Cevallos-Almeida et al. 2019). The increase in stool scores is linked to the increase in secretions from the epithelium to the lumen of the intestinal tract caused by the invasion of *Salmonella* into enterocytes. This invasion is generally accompanied by an intense acute inflammatory reaction, which causes mucosal damage and destruction of the microvilli, reducing absorption and increasing the output of fluids and electrolytes to the intestinal lumen (Figuroa Ochoa and Rodríguez 2005). These changes in stool consistency from 5 DPI were accompanied by a decrease in daily weight gain or an increase in loss weight in 7 out of the 8 pigs evaluated. Considering that the expected weekly weight gain of healthy pigs of the same age should be between 2 and 5 kg, we observed that experimental *Salmonella* infection had a negative impact on daily weight gain. During the first

week, the average weight gain was close to expected (1.95 kg), but from 6 DPI, and on at least one of the days of the study, most of the pigs lost weight. Therefore, during the second week of the study, the average weight gain was reduced to 0.75 kg. This reduction in daily weight gain values was observed even in pigs that showed no clinical signs of the disease or no shedding of the bacterium in their feces, suggesting that all animals were affected due to the experimental infection with the pathogen. In concordance with this, stool consistency was also affected mainly from 6 DPI, showing the highest scores at 9 DPI, coinciding with the highest levels of *Salmonella* isolation.

Salmonella excretion was not homogeneous after infection. In two animals, the microorganism was detected the day after the experimental infection, coinciding with the study by Hurd et al. (2008), who detected the pathogen at high concentrations in fecal samples within a few hours of infection. However, the highest levels of excretion were observed at 9 DPI. These results differ from those reported by other authors, who observed the highest excretion percentages during the first days after infection (Boyen et al. 2009, Cevallos-Almeida et al. 2019). In 3 of the pigs studied, *Salmonella* excretion was intermittent throughout the study. Intermittent excretion of *Salmonella* has been previously described in naturally infected pigs (Pires et al. 2013) and after experimental infection (Boyen et al. 2009, Cevallos-Almeida et al. 2018). This excretion characteristic of the microorganism may be due to the fact that infected pigs go through stages of active excretion and no excretion during the entire period of infection. Even many times, the microorganism can be excreted at levels below the detection limit (Lahodny et al. 2017). In pigs in which intermittent excretion of the bacterium was observed, the presence of the microorganism was detected only in the intestinal tract and/or in the mesenteric lymph nodes together with the presence of lesions.

The colonization and invasion of *Salmonella* in the organs analyzed was variable in the different individuals studied. In the different sections of the gastrointestinal tract, *Salmonella* was isolated mainly in the distal portions (ileum, cecum and colon), presumably due to the lower levels of pH and bile salts compared with those in the duodenum and jejunum (Cevallos-Almeida et al. 2018). In the case of mesenteric lymph nodes, which are considered one of the organs from which the bacteria can most usually be isolated, the pathogen was isolated in 50% of the pigs studied. The results obtained agree with those of most of the authors who consider the tonsils, mesenteric lymph nodes and gastrointestinal tract as the most relevant sites for the colonization and invasion of *Salmonella*, including *S. Typhimurium* (Naberhaus et al. 2020).

As described in the results section, a change in the macroscopic morphology was observed in the *Salmonella* colonies re-isolated from the internal organs. This is possibly due to the modification or partial loss of the bacterial cell wall since, once *Salmonella* invades, it can remain viable within the host cells in the L-form as a spheroplast. This phenomenon is often induced by certain components of the serum, such as antibodies and the complement system (Porres 1973). This type of phenotypic changes may also be associated with the differential expression of pathogenicity

and survival attributes, such as adhesion fimbriae, the production of outer membrane proteins or changes related to the evasion of the immune response (Figueroa Ochoa and Rodríguez 2005).

After the experimental infection with *Salmonella*, we observed epithelial damage and inflammation of the intestinal mucosa, mainly of the ileum, in agreement with that observed by other authors (Schultz et al. 2017, Naberhaus et al. 2020). The fact that the ileum is the sector with the highest colonization by *Salmonella* may be due not only to the immune response caused by the bacterium in Peyer's patches (Argüello et al. 2018), but also to the presence of short chain fatty acids produced by the commensal microbiota, mainly acetate, propionate and butyrate. In the ileum, there are higher concentrations of acetate, which induce the expression of genes present on the pathogenicity island 1, allowing invasion of the ileum mucosa. On the other hand, propionate and butyrate are present in higher amounts in the colon and in the cecum, causing an antimicrobial effect, reducing the expression of the same invasion genes (Schultz et al. 2017). Generally, the deterioration of the intestinal barrier and the imbalance of the intestinal microbiota allow the spread of *Salmonella* to internal organs (Schultz et al. 2017, Naberhaus et al. 2020). Several authors have described the predilection of *Salmonella* for the liver and lymphoid organs such as the spleen, mesenteric lymph nodes and tonsils, causing lesions in them (Figueroa Ochoa and Rodríguez 2005), as observed in this study. In the animals in which microscopic lesions were observed in the intestine, it was possible to re-isolate *Salmonella* Typhimurium from different portions of the gastrointestinal tract. Likewise, in the animals that presented microscopic lesions in different internal organs, it was also possible to re-isolate the bacterium from some of them. In a single animal, we observed a relationship between the systemic invasion of *Salmonella* and a decrease in weight loss. We also observed a relationship between the modification of the stool score, lower daily weight gain, and presence of lesions in the intestine.

In general, IgA antibodies were detected in the ileum mucosa in all pigs. The highest levels of this isotype were observed in pigs in which the bacterium was isolated from the mesenteric lymph nodes. This suggests that, in these pigs, there was a greater stimulation of the antibody-producing cells in this effector organ. Among the antibody responses against *Salmonella*, the presence of IgA in the intestinal mucosa is one of the most relevant, since it has been shown that these antibodies have the ability to reduce the adhesion of the bacterium to the intestinal epithelium and thus prevent the entry into the lamina propria (Huus et al. 2021). In addition, although the action of circulating antibodies against *Salmonella* infection can be variable and although the levels of detectable antibodies can vary during the infection period and can be low or absent even among pigs that are infected and live in the same space (Schmidt et al. 2021), in the present study, we were able to determine the levels of circulating IgG and IgA antibodies in the blood. Serum IgG and IgA levels were relatively similar in all pigs, regardless of the detection of the pathogen in the different organs of each animal. Regarding the agglutinating antibodies in the serum, in general, most of the animals

presented low titers (between 1:4 and 1:8), except one of the pigs, which presented relatively high binding antibody titers. In this animal, the bacterium was only isolated from the ileum, which would suggest that these agglutinating antibodies could have opsonized the bacterium, reducing its invasive capacity (Bioley et al. 2017).


Considering the isolation of the bacterium in the different organs analyzed, two infection patterns were observed. Four pigs presented intermittent excretion of *Salmonella* in their feces, and the bacterium was only re-isolated from different sections of the intestine, mainly in the ileum and the cecum, and/or in the mesenteric lymph nodes, suggesting an enteric infection. On the other hand, the 4 remaining pigs in which the excretion of the pathogen was also variable, the bacterium was re-isolated both in the internal organs and in the different portions of the intestinal tract, which suggests that, in these animals, the infection was enteric and also systemic.

CONCLUSION

In this work, we were able to evaluate the excretion, colonization of organs and histological lesions of a strain of *Salmonella* Typhimurium through the 13 days of the trial. The results obtained allowed a better understanding of the pathogenesis of the disease caused by this pathogen. The observation of two different infectious pictures showed the ability of this *Salmonella* serovar to colonize the gastrointestinal tract and invade internal organs. The data obtained are useful to determine the optimal times to take fecal samples and the number of animals needed in future experimental infection studies. When using this type of infection models for the evaluation of strategies and treatments for its control, it is necessary to take into account the complexity of the pathogenesis of the microorganism.

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REFERENCES

- Argüello H, Estellé J, Zaldívar-López S, Jiménez-Marín Á, Carvajal A, López-Bascón MA, Crispie F, O'Sullivan O, Cotter P, Priego-Capote F, Morera L, Garrido J. Early *Salmonella* Typhimurium infection in pigs disrupts microbiome composition and functionality principally at the ileum mucosa. *Sci. Rep.* 2018; 8(1): 1-12.

2. Audisio MC, Terzolo HR. Virulence analysis of a *Salmonella* Gallinarum strain by oral inoculation of 20-day-old chickens. *Avian dis.* 2002; 46(1): 186-191.
3. Bagul CD, Bodake NL, Pethkar AV. Standardization of ELISA assay for detection of *Salmonella enterica*. *J. Environ. Res. Develop* 2018; 13 (2): 169-174.
4. Bioley G, Monnerat J, Lötscher M, Vonarburg C, Zuercher A, Corthésy B. Plasma-derived polyreactive secretory-like IgA and IgM opsonizing *Salmonella enterica* Typhimurium reduces invasion and gut tissue inflammation through agglutination. *Front. Immunol.* 2017; 8: 1043.
5. Boyen F, Pasmans F, Van Immerseel F, Donne E, Morgan E, Ducatelle R, Haesebrouck F. Porcine *in vitro* and *in vivo* models to assess the virulence of *Salmonella enterica* serovar Typhimurium for pigs. *Lab. Anim.* 2009; 43(1): 46-52.
6. Boyle EC, Bishop JL, Grassl GA, Finlay BB. *Salmonella*: from pathogenesis to therapeutics. *J. of bacterial Res.* 2007; 189 (5): 1489-1495.
7. Bustos CP, Leiva CL, Gambarotta M, Guida N, Chacana PA. *In vitro* Inhibitory Activity of IgY Antibodies Against *Salmonella* Ser. Newport Isolated from Horses. *J. Equine Vet. Sci.* 2021; 103: 103657.
8. Cevallos-Almeida M, Houdayer C, Rose V, Bailly Y, Paboeuf F, Fablet C., Kerouanton A. Colonization of pigs experimentally infected with a monophasic variant of *Salmonella* Typhimurium. *Foodborne Pathog. Dis.* 2018; 15 (9): 576-582.
9. Cevallos-Almeida M, Martin L, Houdayer C, Rose V, Guionnet JM, Paboeuf F, Denis M, Kerouanton A. Experimental infection of pigs by *Salmonella* Derby, *S. Typhimurium* and monophasic variant of *S Typhimurium*: Comparison of colonization and serology. *Vet Microbiol.* 2019; 231: 147-53.
10. Colello R, Ruiz MJ, Padín VM, Rogé AD, Leotta G, Padola NL, Etcheverría AI. Detection and characterization of *Salmonella* serovars in the production chain of two pig farms in Buenos Aires province, Argentina. *Front. Microbiol.* 2018; 9: 1370.
11. European Food Safety Authority and European Centre for Disease Prevention and Control. The European Union One Health 2021 Zoonoses Report. *EFSA Journal* 2022; 20(12), e07666.
12. Figueroa Ochoa IMF, Rodríguez AV. Molecular mechanism for pathogenicity of *Salmonella* sp. *Rev. Latinoam. Microbiol.* 2005; 47 (1-2): 25-42.
13. Hofacre CL, Rosales AG, Da Costa M, Cookson K, Schaeffer J, Jones MK. Immunity and protection provided by live modified vaccines against paratyphoid *Salmonella* in poultry — An applied perspective. *Avian dis.* 2021; 65(2): 295-302.
14. Hurd HS, Gailey JK, McKean JD, Rostagno MH. Rapid infection in market-weight swine following exposure to a *Salmonella* Typhimurium-contaminated environment. *Am. J. Vet. Res.* 2008; 62(8): 1194-1197.
15. Huus KE, Petersen C, Finlay BB. Diversity and dynamism of IgA- microbiota interactions. *Nat. Rev. Immunol.* 2021; 21: 514-525.
16. International Standard Microbiology of food and animal feeding stuffs – horizontal method for the detection of *Salmonella* Spp. ISO 6579:2002.
17. Jajere SM. A review of *Salmonella enterica* with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. *Vet. World* 2019; 12 (4): 504-521.
18. Joaquim P, Herrera M, Dupuis A, Chacana P. Virulence genes and antimicrobial susceptibility in *Salmonella enterica* serotypes isolated from swine production in Argentina. *Rev. Argen. Microbiol.* 2021; 53 (3): 233-239.
19. Lahodny Jr GE, Gautam R, Ivanek R. Understanding the effects of intermittent shedding on the transmission of infectious diseases: example of salmonellosis in pigs. *J. Biol. Dyn.* 2017; 11 (1): 436-460.
20. Malorny B, Hoorfar J, Bunge C, Helmuth R. Multicenter Validation of the Analytical Accuracy of *Salmonella* PCR: towards an International Standard. *Applied and Environ. Microbiol.* (2003); 69 (1): 290-296.
21. Mandal BK, Brennan J. Bacteraemia in salmonellosis: a 15-year retrospective study from a regional infectious diseases unit. *BMJ: Br. Med. J.* 1988; 297: 1242.
22. Miles AA, Misra SS, Irwin JO. The estimation of the bactericidal power of the blood. *Epidemiol. Infect.* 1938; 38 (6): 732-749.
23. Naberhaus SA, Krull AC, Arruda BL, Arruda P, Sahin O, Schwartz KJ, Burrough ER, Magstadt DR, Ferreyra FM, Gatto IRH, Meiroz de Souza Almeida H, Wang C, Kreuder AJ. Pathogenicity and competitive fitness of *Salmonella enterica* serovar 4,[5], 12: i- compared to *Salmonella* Typhimurium and *Salmonella* Derby in swine. *Front. Vet. Sci.* 2020; 6: 502.
24. Nair D, Venkitanarayanan K, Kollanoor Johny A. Antibiotic-resistant *Salmonella* in the food supply and the potential role of antibiotic alternatives for control. *Foods* 2018; 7(10): 167.
25. Okamoto AS, Andreatti Filho RL, Lima ET, Pereira REP, Menconi AIII, Rocha TS, Marietto-Gonçalves GA. Immunological evaluation of the intestinal mucosa of broiler chicks treated with *Lactobacillus* spp. and challenged with *Salmonella* Enteritidis. *Braz. J. Poultry Sci.* 2007; 9(4): 259-262.
26. Olivieri MAC, Moyano RD, Traveria GE, Pinedo MFA, Mon ML, Gravisaco MJ, Delgado FO, Santangelo MP, Romano MI. Protection efficacy of Argentinian isolates of *Mycobacterium avium* subsp. paratuberculosis with different genotypes and virulence in a murine model. *Vet. Sci. Res. J.* 2018; 121: 4-11.
27. Pedersen KS, Holyoake P, Stege H, Nielsen JP. Observations of variable inter-observer agreement for clinical evaluation of faecal consistency in grow-finish piglets. *Prev. Vet. Med.* 2011; 98(4): 284-287.
28. Pires AFA, Funk JA, Bolin CA. Longitudinal study of *Salmonella* shedding in naturally infected finishing pigs. *Epidemiol. Infect.* 2013; 141(9): 1928-1936.
29. Porres JM. Esferoplastos, protoplastos y formas L bacterianas: Su posible papel en el proceso infeccioso. *Rev. Fac. Med. Univ. Navarra.* 1973; 311-324.
30. Rincón-Gamboa SM, Poutou-Piñales RA, Carrascal-Camacho AK. Antimicrobial resistance of Non-Typhoid *Salmonella* in meat and meat products. *Foods* 2021; 10(8): 1731.

31. Schmidt S, Kreutzmann H, Stadler M, Mair KH, Stas MR, Koch M, Vatzia E, Dürlinger S, Knecht C, Spergser J, Dolezal M, Springer S, Theuß T, Fachinger V, Ladinig A, Saalmüller A, Gerner W. T-Cell Cytokine Response in *Salmonella* Typhimurium-Vaccinated versus Infected Pigs. *Vaccines* 2021; 9(8): 845.
32. Schultz BM, Paduro CA, Salazar GA, Salazar-Echegarai FJ, Sebastián VP, Riedel CA, Kalergis AM, Lobos MA, Bueno SM. A potential role of *Salmonella* infection in the onset of inflammatory bowel diseases. *Front. Immunol.* 2017; 8: 191.
33. Underwood W, Anthony R, Cartner S, Corey D, Grandin T, Greenacre C, Gwaltney-Brant S, McCrackin MA, Meyer R, Miller D, Shearer J, Yanong R. AVMA guidelines for the euthanasia of animals: 2013 edition. Schaumburg, IL: American Veterinary Medical Association.

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