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

Joaquim, P.1*; Balbiani, F.1; Delgado, F.1; Redondo, L.1,3; Cappuccio, J.2,3; Chacana, P.1,3

1Instituto de Patobiología-UEDD IPVET INTA CONICET, Centro de Investigación en Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuaria, Argentina. 2Estación Experimental Agropecuaria Marcos Juárez, Instituto Nacional de Tecnología Agropecuaria, Argentina. 3Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina. joaquim.patricia@inta.gob.ar

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 patogenicidad 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 are 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 CienciasVeterinarias 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 x 10⁹ 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 x 10⁹ 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 10⁶ 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.

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<th>Days Post-infection</th>
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*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.

<table>
<thead>
<tr>
<th>Gastrointestinal and internal organs</th>
<th>Pig 1</th>
<th>Pig 2</th>
<th>Pig 3</th>
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<th>Pig 6</th>
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<td>Ileum</td>
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<td>Mesenteric lymph nodes</td>
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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.
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).

<table>
<thead>
<tr>
<th>Pig</th>
<th>Slow plate agglutination (Titer)</th>
<th>ELISA - LPS IgG in serum (OD)</th>
<th>ELISA - LPS IgA in serum (OD)</th>
<th>ELISA - LPS IgA in the mucosa of the ileum (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:8</td>
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<td>0.0631</td>
</tr>
<tr>
<td>2</td>
<td>1:4</td>
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<td>0.6986</td>
<td>0.0790</td>
</tr>
<tr>
<td>3</td>
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<td>0.5347</td>
<td>0.9740</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
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<td>1:8</td>
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<td>0.5071</td>
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<td>0.7035</td>
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</tbody>
</table>
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 (Lahodyn 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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**ORCID**

Joaquim, P. https://orcid.org/0000-0001-8961-7565
Redondo, L. https://orcid.org/0000-0001-6785-2812
Cappuccio, J. https://orcid.org/0000-0002-9256-887X
Chacana, P. https://orcid.org/0000-0003-2824-8385

**REFERENCES**

Inhibitory Activity of IgY


