Two In Vivo Models to Study Salmonella Asymptomatic Carrier State in Chicks

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Running head: Experimental Salmonella *infection models in chicks*

Abstract

In chicken, *Salmonella* Enteritidis and *Salmonella* Typhimurium, the two main serotypes isolated in human infections, can persist in the host organism for many weeks and up to many years without causing any symptoms. This persistence generally occurs after a short systemic infection that may either lead to death of very young birds or develop into cecal asymptomatic persistence, which is often accompanied by a high level of bacterial excretion, facilitating *Salmonella* transmission to counterparts. Here we describe two models of chick infection. The first model reproduces well the poultry infection in farm flocks. Numerous reinfections and animal-animal recontaminations occur leading to a high level of cecal colonization and fecal excretion in all chicks in the flock, over several weeks. In the second model, these animal reinfections and recontaminations are hampered leading to heterogeneity of infection characterized by the presence of low and super-shedders. This model allows more mechanistic studies of *Salmonella/*chicks interactions as animal recontaminations are lowered.

Key words

Salmonella infection, chicken models, heterogeneity of infection, animal-animal transmission, carrier state.

1 Introduction

Salmonellosis is of particular concern regarding economic losses to the livestock industry and threats to human health throughout the world. *Salmonella* is the second cause of human food-borne illness, causing substantial mortality and economic losses (1). Depending on the host and serotype, *Salmonella* has the ability to cause a wide range of diseases including lethal systemic infections and asymptomatic infections. Most serotypes can cause gastroenteritis in humans, which is often uncomplicated and does not need treatment, but disease can be severe in the young, the elderly, and patients with weakened immunity. Centers for Disease Control and Prevention (CDC) estimate *Salmonella* causes 1.35 million infections, 26,500 hospitalizations, and 420 deaths every year in the United States. Contamination of humans mainly occurs through consumption of contaminated pork and chicken. In pigs and chickens, non-typhoidal *Salmonella* serovars may induce a systemic infection in young animals, potentially leading to death, but are more frequently responsible for a long-term asymptomatic infection often referred to as carrier state (2). *Salmonella*-carrier animals are a serious issue as the animals often excrete high levels of bacteria in their feces and may therefore contaminate their congeners but also meat products during slaughter. Prevention of human non-typhoidal salmonellosis thus depends on decreasing the prevalence of infections in livestock hosts and on identifying and intervening along key transmission routes. However, effective control strategies require an improved understanding of the dynamics of infection within host populations.

To analyze the asymptomatic carrier state induced in chickens by *S.* Enteritidis, the most frequently isolated serovar in this host, we therefore developed a first experimental carrier state model. To this end, we inoculated *S.* Enteritidis orally in white leghorn chicks, reared in cages, with the aim to reproduce as closely as possible the carrier-state observed in flocks. At a low dose ($1x10^2$ cfu/chick), *S.* Enteritidis inoculated at 1 day of age induces a high

mortality rate, whereas inoculation of $5x10^4$ cfu/chick at 3 weeks of age does not induce a high level of contamination or persistence (3). This led us to choose the low dose of $5x10^4$ cfu/chick inoculated at 1 week of age for our model (4). With this model, no death or organ lesions were observed subsequent to oral infection. The challenge strain was present in most animals at measurable levels for three to four weeks in the liver and spleen and at high levels for around 10 weeks in ceca, depending on the genetics of the host (4, 5). Moreover, these *Salmonella*-carrier animals also excreted high levels of *Salmonella* in their feces and they represented a significant reservoir with the potential to contaminate neighboring naïve animals and also the environment. This first model, called the "cage model" in which chickens are housed above ground on a slatted floor, mimics well the contamination observed in poultry reared in flocks. However, it does not allow the mechanisms of *Salmonella*/chick interactions to be elucidated easily as there is a transmission of the pathogens between birds through contaminated air particles and highly contaminated fecal samples (*see* **Note1**). To determine if *Salmonella*-carrier state corresponded to persistence in ceca or was due to a constant recontamination of chickens, we developed a second model of chick infection in isolators ("isolator model") in which animal reinfection and host-to-host transmission are greatly decreased.

The results regarding *Salmonella* colonization and excretion levels between the two models of infection are very different. The hallmark of the "cage model*"* is the high level of cecal colonization observed in all chicks for several weeks, with quite low inter-animal variability. Similarly, a high level of excretion was obtained, when we collected fresh fecal samples. The hallmark of *Salmonella* infection in the "isolator model" is that, in these reduced recontamination conditions, *S.* Enteritidis induces a heterogeneous infection (6). Heterogeneity of infection is characterized by the presence of some chicks known as low-shedders because they excrete no or few bacteria in their feces and have a low cecal

colonization level. In contrast, others, called super-shedders, have high cecal colonization and excrete high levels of *Salmonella*, which are very close to levels observed in the "cage model". Other chicks have an intermediate phenotype. This heterogeneity of cecal colonization and fecal excretion was observed both when only one chick per isolator was infected and when 30 chicks were infected in a large isolator (6). The comparison of the two models of infection shows that, in cages, the high level of *Salmonella* excretion observed in all chicks is obtained 7 days post inoculation, whereas in isolators some chicks start to excrete *Salmonella* only after 14 days, even when they are alone in an isolator. The presence of super-shedders, under conditions of low host-to-host transmission or reinfection, demonstrated that constant reinfection was not required to induce a carrier-state at the animal level. However, these results show that homogeneity of *Salmonella* colonization and excretion, observed in cages, is most likely related to the presence of super-shedders, which constantly disseminate *Salmonella* to the low-shedder chicks. This transmission occurs via feed and water contaminated by feces and through airborne movements of contaminated dust particles (6). One should note that the airborne transmission is sufficient to infect healthy chicks reared in a cage far from another cage containing super-shedder chicks. Moreover, the *Salmonella* colonization pattern obtained by airborne transmission is similar to that observed after an oral contamination in our cage model (7).

A fine analysis of the causes linked to the appearance of the low- or super-shedder phenotypes showed that low-shedder chicks do not have a higher capability to destroy *Salmonella* but instead can block initial *Salmonella* colonization partly due to the presence, before infection, of specific gut microbiota taxonomic features (8). In conclusion, susceptibility to *Salmonella* infection is a multi-factorial process, which involves the gut microbiota, which is the first barrier to *Salmonella* colonization. The genetics of the host, and the innate and adaptive

immune response, which also play determinant roles, are mainly involved in the level of intestinal and systemic infection and in the elimination of the pathogen (8) (9) (10).

Here, we describe the two chick models, which allow the different mechanisms of *Salmonella* colonization in chickens to be deciphered. In particular, we describe the critical points of the protocols, which determine whether chicks develop either a homogeneous colonization or a heterogeneous infection.

2 Materials

2.1 Bacterial strain

A spontaneous streptomycin-resistant clone of the nalidixic acid-resistant *Salmonella* Enteritidis LA5 strain was obtained in our laboratory. This well-characterized parent strain was isolated from a 4-day-old broiler chicken (11) (12). (*see* **Note 2**)

2.2 Inoculum preparation

1. 1L Erlenmeyer.

2. TSB (Tryptic Soy Broth) medium

3. 500 µg/mL Streptomycin

4. 20 µg/mL Nalidixic acid

5. 1.8mL cryogenic vials.

6. 50% Glycerol solution in saline water (0.85% NaCl).

7. Rappaport medium (*Salmonella* selective medium)

8. Rambach medium

9. TSA medium

10. Salmonella Shigella medium

11. PBS

12. TH5 disinfectant

2.3 Animals

White Leghorn, Specific Pathogen Free (SPF), line PA12 (INRAE-PFIE origin), 7-day-old, female and male chicks. Bird banding is performed before the start of the experiment in order to follow the animals individually.

2.4 *In vivo* experiment

1. Feed

The same feed with the same composition and granulometry is used for all the experiments, whatever the age of chickens (*see* **Note 3**). This feed is prepared by our institute (INRAE, UE PEAT Centre Val de Loire, 37380 Nouzilly, France). The feed composition corresponds to: 27.97% corn , 30% wheat, 34.2% soybeans , 4% soybean oil, 0.94% calcium carbonate , 1.8% dicalcium phosphate, 0.17% methionine , 0.4% salt , 0.4% Prémix VHT791NE (Techna France) containing several additives including oligo-elements, vitamins, etc. Depending on the experiment, an anticoccidial can be added, but this will modify the gut microbiota composition and can therefore modulate *Salmonella* colonization. The grain size of the pellets is 2.5 mm in diameter and 4 to 6 mm in length for starter and grower animals.

2. Battery cages

Before introducing chicks in isolators, they are reared in battery cages in the same room. This step allows the chicks to acquire a starting flora and to partially homogenize their intestinal flora. Four tiers of 2.8x.8m cages are equipped with feeders, drinkers and an infra-red lamp to provide additional

warmth to the chicks. Under the living area, there is a tray so that the droppings do not fall on the animals on the tier below. The battery cages can accommodate 350 chicks from 1 to 12 days old depending on the lineage. The temperature is 35°C for the first week and then it gradually decreases (32°C week 2 ; 28°C week 3 ; 25°C week 4 ; 20°C week 5 ; 18°C week 6). The hygrometry is 30 to 40% when the temperature is 37°C. It increases to 70% when the temperature is between 22 and 24°C. It fluctuates with the outside hygrometry.

3. Cage

In this model, chickens are housed above ground on a slatted floor (Fig. 1). As *Salmonella* can be transmitted via air borne particles (7), each cage is installed in a separate room with the same environmental conditions (feed, water, temperature, air humidity and lighting scheme). All rooms are equipped with a high efficiency particulate air filter (HEPA, H13). The surface of the cages can be modulated, depending on the number of chicks, with a maximum of 4 m^2 which is designed for 150 chickens, depending on the age and the lineage. Chicks of the same group are reared in a cage on slatted flooring, which is covered with wire mesh: 1 cm x 1cm for chicks up to 10 days of age, and 2 cm x 2 cm for chicks over 10 days of age. Under these conditions, only a small quantity of feces is present on the floor.

All chicks have free access to drinking water and are fed ad libitum. Between days 1-7, a sheet of craft paper with an infra-red lamp suspended in the cage to keep the chicks at a temperature of 32°C. They are kept under the infra-red lamp for 28 days (Table 1). See table below. A 12:12 L:D lighting scheme is applied.

4. Large isolator

A type A3 confined isolator (Fig. 2) of 2.26 m^2 is used. In the isolator, host-to-host transmission between chicks and subsequent reinfections are severely hampered through efficient filtration of air with HEPA filters (H14) at the inlet and outlet. An H7 pre-filter is positioned at the extraction side to trap large particles such as dust. The air change rate is above 54 m^3/h. The temperature is recorded by sensors and modified by the room temperature control program, depending on the age of the animals. If initially the battery cages cannot be used, for the first few days a sheet of craft paper and an infrared

lamp on top are inserted into the isolator to keep the chicks at a temperature of 32°C. This infrared lamp can be kept for up to 15-21 days to obtain a temperature of around 23°C. The hygrometry is 30 to 40% when the temperature is 37°C. It increases to 70% when the temperature is between 22 and 24°C. It fluctuates with the outside hygrometry. The isolator consists of three parts: 1-a handling area where feed and materials are stored. A liquid airlock containing a disinfectant (TH5 at 2%), which neutralizes the pathogenic agent, allows animals or biological samples to be introduced or removed via leak-proof containers without breaking containment. 2- An accommodation room where feed is suspended above floor level in order to limit direct contamination through animal droppings. The drinking water is delivered by a pipette system with small drinking troughs. Contact with the droppings is limited as a grid allows the droppings to fall into a receiving tray containing a decontaminant solution of quaternary ammonium which results in quick sterilization. The wire mesh of the grid is 1 cm x 1cm for chicks up to 10 days of age, and 2 cm x 2 cm for chicks over 10 days of age. A removable partition with a guillotine door separates the breeding space and facilitates handling. 3- An excretion pit located under the breeding space must be regularly decontaminated with a germicide (TH5 2%) (*see* **Note 4**).

All chicks have free access to drinking water and are fed *ad libitum*. A nycthemeral rhythm of 12 hours of darkness and 12 hours of light is applied. The isolator is equipped with five pairs of rubber gloves for handling.

5. Inoculation

5.1 I.V. Catheter Terumo® (22Gx1).

5.2 Syringe Plastipak 1mL luer lock,.

5.3 Needle Terumo® (25Gx5/8", 0.5x16mm)

6. Euthanasia

6.1 Circlip pliers for chicks younger than 15 days.

6.2 Paper towel

6.3 Aluminum foil

6.4 Bunsen burner

6.5 A poultry stunner (VE memory in our case) and a home-made guillotine

7. Autopsy

7.1 1.8 mL cryogenic vials.

7.2 Dry ice

7.3 Pre-weighed plastic sample pots (1/droppings)

7.4 Dissection kit with scissors and surgical clips

7.5 A beaker with 70° alcohol for each manipulator

7.6 Pre-weighed blender bags with filter (1/droppings or ceca or internal organs)

7.7 Sterile Eppendorf microtubes

7.8 RNAlater (Qiagen) or equivalent product

7.9 70° alcohol

7.10 Dry ice

7.11 PBS containing 0.1%Tween80

7.12 0.14 mol/mL EDTA (ethylene diamine tetra-acetic acid)

2.5 Bacterial numeration

1. 150 mm Petri dishes containing selective medium Salmonella-Shigella (SS) agar supplemented with antibiotic, 500µg/mL streptomycin sulfate salt (Sm500), 20 µg/mL Nalidixic acid (Nal20).

2. Bagmixer (e.g. MiniMix CC, Interscience).

3. Automatic plater (e.g. easySpiral Pro, Interscience) or dilution tubes with 1.8 mL saline water.

4. Automatic colony counter (e.g. Scan 4000, Interscience).

5. PBS

3 Methods

3.1 Preparation of a frozen inoculum

1. Grow the *Salmonella* strain in 10 mL of trypticase soya broth supplemented with the appropriate antibiotic overnight at 37°C with agitation (190 rpm).

2. The next day, inoculate 400 mL of TSB supplemented with the appropriate antibiotic with 4 mL of the overnight pre-culture and incubate the Erlenmeyer for 24h at 37°C under agitation (190 rpm).

3. Centrifuge the bacterial culture at 4,800 g for 20 minutes at 20°C and resuspend the pellet in 20 mL of PBS containing 50% glycerol.

4. Distribute 1.2 mL of the obtained solution in 1.8 mL cryogenics vials and store them at -80°C.

5. A few days after freezing, unfreeze one vial and numerate the inoculum to determine the exact concentration of the inoculum by serial dilutions in PBS and spread on TSA plates. This vial is thrown away after use.

3.2 Animal handling before inoculation

Animal experiments must be carried out in strict accordance with the local legislation, and the experiments must be approved by an ethics committee. The principles of reduction, replacement and refinement are implemented in all animal experiments. Everybody working on animals should be allowed to handle animals.

1. For the cage model: Just after hatch, all chicks are reared together in the cage for 6 days to favour acquisition and homogenisation of the gut microbiota. Then the 6-day-old chicks are randomly distributed into the different cages according to the different conditions.

2. For the isolator model: from the day of hatch, chicks are reared together in the battery cages at a temperature of 35°C for 6 days to favour acquisition and homogenisation of gut microbiota. If chicks receive a treatment or bacteria the day of hatch, each group is reared in a separate room. The method of transferring chicks depends on the type of isolator. We used a box to transfer the chicks through the airlock or the double watertight door.

3.3 Checking for the absence of *Salmonella* in chicks

The EOPS status of the animals is checked regularly. However, before the experiment the *Salmonella*-free status of the birds is verified. For this purpose:

- Place wipes in the transport boxes of the chicks.
- After transport, place the dropping-stained wipes in 10 mL buffered peptone water for 24 hours at 37°C.
- Prepare a 1/10 dilution of this peptone water in Rappaport medium. Incubate for 24 hours at 37°C.
- Isolate bacteria on Petri dishes containing Rambach medium (*Salmonella* colonies are fuschia red in color).

3.4 Inoculation

1. On the day of inoculation (7-day-old chicks), unfreeze one inoculum aliquot and dilute it to adjust the bacterial concentration to 2.5×10^5 CFU/mL before inoculation. Prepare at least 50% more inoculum than the volume you need to inoculate the chicks. To standardize the different experiments in the same project, one frozen aliquot from the same initial inoculum preparation is used for each experiment. The aliquot is thrown away after use.

2. Numerate this preparation in order to validate its 2.5×10^5 CFU/mL final concentration. Numeration can be performed on an automatic plater (easySpiral Pro) or by serial dilutions of the inoculum in PBS.

3. Orally inoculate 0.2 mL of the 2.5×10^5 CFU/mL suspension with a 1 mL syringe mounted with a catheter at the end. To do this, one person holds the chick while another person stretches its neck slightly and opens the beak by pressing on the base of the beak, in order to enter the catheter into the esophagus without hurting the chick, and injects 0.2mL of the suspension (Fig. 3) (*see* **Note 5**).

3.5 Sample recovery from live animals

For chicks reared in cages or isolators several samples are recovered before and after infection. The type of samples and when they are retrieved may vary depending on the scientific question but generally, samples are taken at 4, 6, 11, 14, and 21, 28 days of age (*see* **Note 6**).

1. Fresh fecal samples:

Colonization levels of the *Salmonella* strain and the gut microbiota composition can be studied on fecal samples.

- Collect fecal samples by gently pressing the chick abdomen (*see* **Note 7**). Droppings are collected in a pre-weighed sampling pot.
- A small amount of droppings is deposited quickly in a sterile Eppendorf microtube using a sterilized single use spatula and rapidly frozen in a dry ice/alcohol bath for further analysis of the microbiota.
- The rest of the feces in the pot is conserved for bacterial numeration.

It is advisable to collect droppings as soon as the lighting is switched on because the chicks feed and expel feces immediately after the lights come on.

2. Blood samples:

Blood is recovered in the occipital sinus to measure the number of circulating immune cells, levels of cytokines, and gene expression of immune cells.

The chick is kept upright with its head bent forward. Blood is taken using a syringe equipped with a needle and a guard allowing the sample to be taken at the level of the occipital sinus, an arteriovenous node located at the back of the head, which allows a larger volume to be taken in a few seconds without risk of hematoma, compared to sample taken from the artery under the clavicle. The guard allows the needle to be inserted at a constant distance to preserve the chick and to be sure to enter the vein. For chicks under 6 weeks old, the needle should not exceed 3mm from the guard. The guard corresponds to the cap of a 25Gx5/8" (0.5x16mm) needle with a length of 26 mm.

3.6 Post-mortem sample recovery

1. Euthanasia

- Chicks under 15 days old are killed using circlip pliers. The neck of the chick is placed between each arm of the plier and the cervical vertebrae are broken.
- Over 15 days of age, chicks are first stunned by electronarcosis before crushing the cervical vertebrae with a guillotine.

- After death, chicks are rapidly soaked in a warm quaternary ammonium solution (Lukewarm, 1/2000 for few a minutes) in order to disinfect the outer surface of the animal.

2. Beginning of the necropsy

- Install the dissection station. Light the Bunsen burner, fill the beaker with 70% alcohol (up to the height of the scissor blades) and put the scissors and surgical clips in the beaker. Install a paper towel on a piece of aluminum foil close to the Bunsen burner (Fig. 4A).
- Take a chick from the quaternary ammonium solution and drain off excess liquid. Then put it on its back on the paper towel.
- During the autopsy, scissors and clips must be sterilized in the flame of the Bunsen burner and left to cool slightly before each use.
- First, the chicken skin is removed by making an incision in the lower abdomen and by lifting the skin up to the neck (Fig. 4B). Then soak the scissors and surgical clips in 70% alcohol, put them in the flame and wait for them to cool.
- Second, open the chick, incise the muscle just under the tip of the wishbone and cut along the wishbone (Fig. 4C). Again, sterilize the scissors and surgical clips in 70% alcohol and Bunsen burner flame, and let them cool.
- Cut the wishbone to facilitate organ removal and sterilize scissors and surgical clips once again. In addition, pulling the thighs backwards breaks the connection at the spine, thus keeping the thighs apart and making it easier to take samples (Fig. 5A).

3. Blood sampling

It is possible to take a blood sample during autopsy. Blood recovered from the heart post-mortem should be used more for antibody research than for working on viable cells.

- Soak Pasteur pipettes in a beaker containing 0.14 mol/L EDTA to prevent blood clotting in the pipette.
- Introduce the pipette equipped with a pear-propipette into the heart, quickly after opening the animal. Let the blood rise in the pipette.
- Put the blood into a sterile Eppendorf tube containing 100µL of 0.14 mol/L EDTA.

4. Recovery of internal organs

If collected, internal organs should be removed before intestinal organs as the latter could contaminate the former. Systemic *Salmonella* colonization can be estimated in the spleen or liver after necropsy. The liver is burgundy red in color and is located just above the intestine (Fig. 5A). Only take a small piece of liver. It is sufficient and avoids bleeding. The spleen is also often used to study the systemic immune response using qRT-PCR. The spleen is located under the proventriculus (Figure 5B). It is small, round and garnet red in color. To recover the spleen, lift the gizzard.

Liver and spleen samples are each put in a pre-weighed and identified blender bag for bacterial enumeration or in a sterile Eppendorf microtube containing RNALater (Qiagen, or equivalent product). The spleen and liver samples can be cut in half to provide samples for the two types of analysis (bacterial numeration and immune response). Samples in RNALater should be conserved at 4°C overnight and then can be stored for several months at -80°C.

5. Cecal tissue and cecal content recovery

Several analyses can be performed on ceca including: *Salmonella* numeration, mucosal immune response analysis (cecal tissue or cecal tonsils), microbiota analysis (cecal content or cecal mucus) and intestinal metabolite analysis (cecal content)

The cecum is located at the junction of the ileum and the colon (Fig. 5B and 5C). Be careful to collect all the cecum by cutting it at its intersection with the intestine. A cecum can be recovered after necropsy to quantify levels of *Salmonella* colonization. This cecum should be collected in a pre-weighed and identified blender bag. A second cecum can be used to collect cecal content (*see* **Note 8**). Cecal content is collected in a pre-weighed sampling pot and some of this sample should be deposited quickly in a sterile Eppendorf microtube with a sterilized single use spatula and rapidly frozen in a dry ice/alcohol bath for further analysis of the microbiota or metabolites.

To collect cecal mucus samples, after recovery of cecal content, the cecal tissue is washed and gently stirred in PBS to remove attached cecal content. Two additional washes are conducted with PBS containing 0.1% Tween 80 to recover mucus. The resulting mixture is shaken vigorously and rapidly frozen in dry ice for further processing.

From the second cecum, the cecal tonsil, a lymphoid organ related to Peyer's patches in mammals, can be collected (Fig. 5C). The cecal tonsil is located at the base of the cecum very close to the intersection of the cecum with the ileum. The cecal tonsil is hardly detected the first days of age but grows rapidly with the age of the chicks. Put the cecal tonsil into a sterile Eppendorf tube containing RNALater (Qiagen, or equivalent product). Samples in RNALater should be conserved at 4°C overnight and then can be stored for several months at -80°C.

3.7 Bacterial numeration

To measure the levels of *Salmonella*, the pre-weighed pot or bag containing the sample (droppings, ceca, spleen or liver) are weighed in order to obtain the weight of each organ (or piece of organ). Add aseptically 2 mL of TSB for spleen or 10 mL for other organs and crush the organ using a Bagmixer (in our case, with the MiniMix CC set at speed 4 for 2 minutes). If necessary, dilutions are performed in PBS. Spreading is carried out on Salmonella-Shigella (SS) medium containing antibiotics in Petri dishes using an easySpiral Pro device. The Petri dishes are then placed at 37°C for 24 hours. Counting is carried out with an automatic counter (Scan 4000 in our case) after adjusting the dilution parameters. The mean counts of *Salmonella* CFU in organs are calculated per gram at each time point. When no colonies are detected, the sample homogenates are enriched to reveal contamination below the detection threshold. Enrichments are performed by diluting each crushed organ in 30 mL tryptic soy broth. After 24h at 37°C, these cultures are plated on SS medium containing nalidixic acid and streptomycin and then incubated at 37°C for 24h. Under these conditions, the detection threshold after enrichment is one bacterium per organ.

4 Notes

1. It is important to take into account that during infection of chickens by *Salmonella*, the ambient air is contaminated. It is therefore essential to wear protective clothing or even take a shower when leaving the room.

2. Antibiotic resistant strains should be used if bacterial numeration is performed on non-sterile organs such as the intestine. A few years ago, we used the LA5 strain but emergence of nalidixic acid-resistant strains in the microbiota of the PA12 chicks forced us to select a streptomycin resistant clone of LA5. The colonization ability/virulence of this clone has been validated.

3. The composition and the granulometry of the feed have an impact on *Salmonella* colonization of chicks. We recommend using feed with a well-defined composition and always the same feed. The composition of different batches of feed supplied by manufacturers can vary.

4. It is not possible to leave a large amount of germicide in the droppings container due to the high humidity level. To kill bacteria present in the fecal droppings under the grids, droppings are covered with the minimal quantity of germicide. This germicide evaporates very quickly, therefore the droppings should be soaked three days a week by watering all around the isolator using a hose connected to a "can" containing the germicide and care should be taken not to leave any germicide on the grids.

5. When inoculating, it is important to wait for the chick to swallow before putting it back on the floor because otherwise the chicks tend to reject part of the inoculum.

6. The time required for the passage of the inoculum into the ileum is variable. This is related to the retention time in the crop and gizzard, which is related to feed intake and can vary from between 0 to more than 12h.

7. For analysis of microbiota composition, it is important to use fresh samples and not fecal samples taken from the floor several hours after dropping. Cloacal swabs often give intermittent presence of *Salmonella* contrary to analysis of fresh fecal samples (4).

8. In the majority of experiments, similar levels of *Salmonella* colonization are observed in the two ceca. Moreover, a higher colonization level is observed in the ceca than in the fresh fecal samples. Similarly, a slightly higher colonization level is observed in the spleen than in the liver.

Table 1: Temperatures used for the cage-reared chickens.

Age (Days)	Temperature under the infrared lamp (°C)	Room temperature (°C)	Height of the 3X150W infrared lamps (cm)	Observations
1-7	32	25	60	Craft paper, feed on floor
7-12	30	25	60	Remove craft paper
12-18	28	23	70	
18-28	25	21	80	
>28		23		Switch off infrared lamp

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Figure captions

Figure 1: Presentation of one cage used in the "cage" model. In this model, chicks are housed above ground on slatted flooring (**A**), which is covered with wire mesh: 1 cm x 1cm for chicks up to 10 days of age, and 2 cm x 2 cm for chicks over 10 days of age. All chicks have free access to drinking water and are fed ad libitum. Between days 1-7, a sheet of craft paper with an infra-red lamp on top suspended is introduced in the cage (**B**).

Figure 1

Figure 2: Presentation of one large isolator used in the "isolator" model. A type A3 confined isolator (**A**) of 2.26m2 is used. It is equipped with five pairs of rubber gloves for handling and it consists of three parts: 1-a handling area where feed and materials are stored. 2- A room for chickens where the drinking water is delivered by a pipette system with small drinking troughs (**B**). 3- An excretion pit located under the breeding space, which must be regularly decontaminated with a germicide.

Figure 2

A B

Figure 3: Chick handling to orally inoculated compounds or pathogens. Chicks are orally inoculated with 0.2 mL of the *Salmonella* suspension with a 1 mL syringe mounted with a catheter on its end. For this purpose, one person holds the chick while another person stretches its neck slightly and opens the beak by pressing on the base of the beak. Enter the catheter into the esophagus without hurting the chick, and inject the suspension slowly.

Figure 3

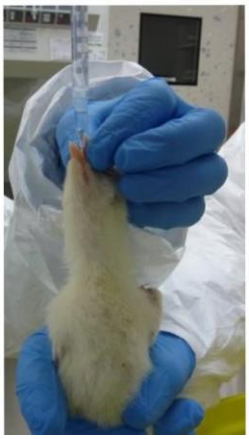

Figure 4: Dissection station and first steps of autopsy. (**A**) The dissection station consists of a large piece of aluminium foil on which is placed a paper towel, a Bunsen burner, a beaker containing 70° alcohol, surgical scissors and clips. (**B**) To open the skin, first the chicken skin is removed by making an incision in the lower abdomen and by lifting the skin up to the neck.

(**C**) Then, an incision of the muscle just under the tip of the wishbone is made and the muscle is cut along the wishbone.

Figure 4

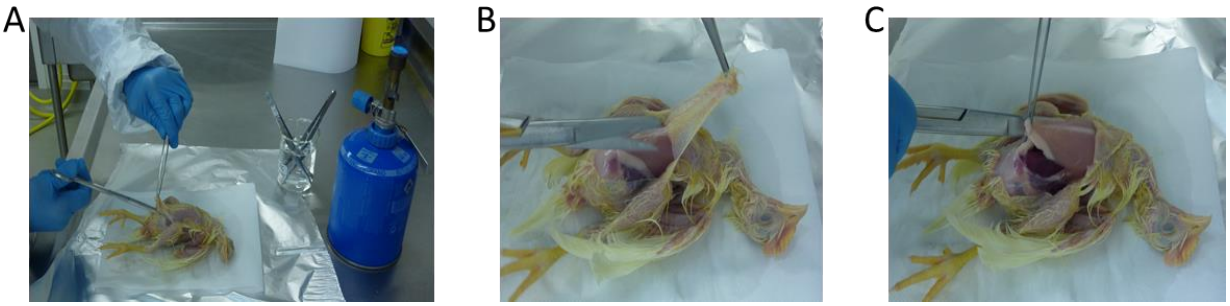

Figure 5: Recovery of organs. Internal organs should be removed before intestinal organs. (**A**) Picture of the chick after opening. The liver is burgundy red in color and is located just above the intestine and the gizzard. (**B**) To see the spleen and the ceca lift the gizzard. The spleen is located under the proventriculus. It is small, round and garnet red in color. (**C**) The ceca are located at the junction of the small and the large intestine. The cecal tonsils, a lymphoid organ related to Peyer's patches in mammals, are located at the base of the cecum very close to the intersection of the cecum with the small intestine.

Figure 5