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Management of Laying Hen Farms in France Infected by *Salmonella* spp.: Comparison of Three Strategies

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Abstract: The presence of *Salmonella* spp. on laying hen farms turns out to be a major problem for public health reasons, mainly related to foodborne illnesses that may arise due to ingestion of contaminated eggs. When *Salmonella* spp. infection on farms is confirmed by laboratory analysis, animal health measures are applied on the establishment, including total elimination of animals. The aim of this study is to describe three different methods of managing *Salmonella enteritidis*-infected laying hen farms, all of which were used within the Moselle department (France). The methods chosen, culling, use of a mobile CO_2 container, or lethal injection with T-61, depend on specific starting conditions, including the number of birds and proximity to an authorized slaughterhouse. This study, therefore, helps to identify the main problems with each method and provides recommendations for improving the management of *Salmonella* spp.-infected laying hen farms, where antibiotics cannot be used.

Keywords: *Salmonella*; laying hen; slaughterhouse; depopulation; antibiotics

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1. Introduction

Salmonella spp. is a facultatively anaerobic, nonsporulating, Gram-negative bacterium belonging to the family *Enterobacteriaceae* [1]. Two major species belong to the genus *Salmonella*: *Salmonella enterica*, which contains six subspecies, and *Salmonella bongori* [2]. According to antigenically diverse surface structures, members of the *Salmonella* spp. can be classified into one of more than 2500 serotypes (serovars) [3]. Regarding salmonellosis in poultry, a distinction must be made between species-specific and non-species-specific *Salmonella* serovars. The species-specific *Salmonella* serotypes are mainly found in one animal species, such as *Salmonella gallinarum* and *Salmonella pullorum*, the agents responsible for typhoid and Pullorum disease [4]. Non-species-specific *Salmonella* serotypes, such as *Salmonella enteritidis* and *Salmonella typhimurium*, can infect several animal species, including humans, and are, therefore, also called zoonotic *Salmonella* serotypes [5]. In addition, they are able to colonize the alimentary tract of poultry without producing symptoms, making the animal hard to identify as risky and facilitating their transmission via table eggs and poultry meat to humans [6].

In humans, *Salmonella* spp. infection has a variable symptomatology that is expressed in vomiting, diarrhea, and fever occurring 48 h after contamination, generally subsiding within 72 h [7]. The development of the disease is favorable about 8 days after the onset of the first symptoms, though immune-depressed patients may have a prolonged and recurrent *Salmonella* spp. infection [7].

Salmonella enteritidis and *Salmonella typhimurium* are a major cause of foodborne illness in Europe; in fact, according to data reported by the European Food Safety Authority (EFSA) in 2022, Salmonellosis is the second most reported foodborne gastrointestinal infection in humans after campylobacteriosis, with 60,050 confirmed cases per 100,000 [8].

The emergence of antimicrobial-resistant *Salmonella* spp. strains due to improper use of antibiotics for therapeutic and prophylactic purposes, and also as growth promoters is a further concern [9]. Nowadays, in keeping with WHO strategic priorities on antimicrobial resistance and with the European Commission guidelines calling for reinforcement of AMR prevention strategies [10,11], the use of antibiotics to control *Salmonella* spp. in poultry is not allowed, except for the exemptions described in art. 2 of Regulation (EC) 1177/2006 [12].

The pharmacovigilance system, which is already used to obtain information on treatments carried out on farms and their outcome, gains further value in the poultry industry by allowing the competent authority to monitor compliance with this ban, and to identify alternative treatment options [13]. The Classy-Farm system, a system of risk assessment and classification on farms developed in Italy, and its checklists for laying hens introduced in 2019, represents a virtuous example of the potential of the pharmacovigilance system thus understood [14].

Since poultry is a major vehicle for transmission of *Salmonella* spp. to humans, several measures need to be implemented to limit the spread of the bacterium, such as adopting biosecurity standards on farms, in addition to reducing the use of antibiotics. European Regulation (EU) No. 429/2016 defines biosecurity as "one of the key prevention tools at the disposal of operators and others working with animals to prevent the introduction, development and spread of transmissible animal diseases to, from and within an animal population" [15]. In France, the 29 September 2021 decree established mandatory biosecurity rules for poultry farms throughout the country [16]. Firstly, the establishment must be re-organized to reduce the risk of introducing *Salmonella* spp., with identifying three distinct areas: (i) a public zone, outside the farm, that may include a visitor reception area and farm accommodation; (ii) a professional zone, outside the breeding area, reserved for the movement of authorized persons and vehicles; and (iii) a breeding zone, consisting of all production units [16].

Before entering the breeding zone, authorized persons must wash their hands and wear specific clothing and footwear; the breeding area must, therefore, be preceded by a zone containing a dressing room and all the equipment needed for disinfection [17]. Moreover, authorized persons must not introduce pets into the breeding zone because they may be carriers of *Salmonella* spp. and may even spread the bacterium [17]. The surroundings of the buildings must be maintained in a satisfactory state of cleanliness; thus, they must include a concrete or stabilized access area to make them easy to clean [16]. A very important factor in farm hygiene management is the control of parasites. Indeed, as they are known to spread *Salmonella* spp. on farms, an owner or keeper must demonstrate that they have a contract or procedure in place for farm-wide control of unwanted rodents and insects [16].

The owner or keeper must monitor all buildings and corridors daily to check the health status of poultry, so as to remove any carcasses that, presenting a high risk of *Salmonella* spp. and other germs contamination, must be enclosed inside a waste container–which must be placed in a public area, and then preferably refrigerated [16,17].

An additional element of risk is feces, which could be positive for *Salmonella* spp. Therefore, storing manure on the farm or spreading it near poultry houses is prohibited [17]. In addition to the biosecurity measures described, it is very important to adopt effective bird management systems, such as the all-in/all-out system, which consists of the entry and exit of all the birds at the same time [16]. There are several advantages to this system, including reduced contact with outsiders and reduced animal stress associated with unloading operations, which reduces the risk of *Salmonella* spp. contamination. In addition, it allows the whole farm to be emptied, cleaned, and disinfected between production periods [17].

Farms must have a control plan for zoonotic agents, including *Salmonella* spp., European Regulation No. 2160/2003 ensures that "proper and effective measures are taken to detect and to control *Salmonella* and other zoonotic agents at all relevant stages of production, processing and distribution, particularly at the level of primary production, including in feed, in order to reduce their prevalence and the risk they pose to public health" [18]. The regulation aims at reducing zoonoses and zoonotic agents on laying hen farms; *Salmonella* serotypes relevant to public health are considered, such as *Salmonella enteritidis*, *Salmonella typhimurium* and the monophasic variant of *Salmonella typhimurium*. Based on this aim, the European Commission adopted European Regulation No. 517/2011 which "implements Regulation (EC) No 2160/2003 of the European Parliament and of the Council as regards a Union target for the reduction of the prevalence of certain *Salmonella* serotypes in laying hens of *Gallus gallus*" [19]. The regulation defines sampling methods for the presence of *Salmonella* spp. in laying hen flocks; these methods are further elaborated in the French decree of the 1 August 2018 [20]. Under this decree, the first sampling is required to take place at a flock-age of 24 $\pm$ 2 weeks or when laying hens are 24 weeks old; thereafter, sampling must be performed every 15 weeks throughout the duration of production. The veterinarian and owner must send the samples to the laboratories within 48 working hours of collection.

Sampling information include: (i) INUAV code (Identifiant Unique Atelier Volailles), (ii) where the group from which the samples were taken is located, (iii) methodologies for rearing, (iv) age of the animals on the date of sampling, and (v) identity of the person who performed the sampling and name of the veterinarian responsible for the sampling [20]. Thereafter, laboratories shall seek to detect *Salmonella* spp. in samples and, in the event of a positive result, serotype at least one isolate from each positive sample [19].

In addition to self-monitoring tests, *Salmonella* spp. detection on farms is also carried out in the following cases: (i) presence of sick consumers after the consumption of contaminated product from the farm, (ii) presence of food samples from the farm that are positive for *Salmonella* spp., (iii) declaration by the farmer where *Salmonella* spp. infection is suspected. In all of these cases, where the suspicion of contamination is confirmed by laboratory analysis, animal health measures will be applied, including the elimination of infected bird flocks [20,21].

The decision to proceed towards elimination is mainly related to the prohibition of antibiotic treatment of the *Salmonella*-infected bird flocks [17]. European Regulation No. 1099/2009 defines depopulation as "the process of killing animals for public health, animal health, animal welfare or environmental reasons under the supervision of the competent authority" [22]. In the specific case of salmonellosis, the decision to proceed with depopulation is linked both to reasons of public health - in order to prevent any food poisoning due to the ingestion of eggs contaminated by *Salmonella* spp.- and to the impossibility of finding an authorized slaughterhouse capable of accepting the batch of contaminated animals. Therefore, when an authorized slaughterhouse can be found that can accept the batch of *Salmonella* spp.-positive laying hens, the poultry owner requires permission from the prefect of the department where the farm is located before dispatching them to the approved slaughterhouse [20]. Instead, where no slaughterhouse can accept the batch of infected laying hens, depopulation is carried out directly on the farm as a function of the number of birds in line with EFSA opinion of 2019 [23]. Thus, the aim of the study is to describe three different methods, and their critical issues, used for the management of *Salmonella* spp.-infected laying hen farms within the Moselle Department (France), given the impossibility of controlling this bacterium with antibiotics.

2. Material and Methods

2.1. First Case

On 19 March 2021, an outbreak of *Salmonella enteritidis* was recorded on a laying hen farm containing 72,000 ISA brown hybrids weighing about 2.5 kg at 70 weeks of age. After confirmation of infection by the National Reference Health Laboratory, animal health measures were implemented and the dispatch of infected animals to an authorized slaughterhouse was considered.

Firstly, due to the absence of a slaughterhouse near the farm capable of accepting the batch of animals, the farmer evaluated the possibility of sending the animals to a neighboring member state (Germany), but difficulties surrounding permissions from the competent authorities in the destination state led them to abandon that plan. Ultimately, the birds were sent to a slaughterhouse located 800 km from the farm, and slaughtering was carried out over several days due to the large number of animals. At the end of operations, the costs to the farmer were around 40,000 euros which included both transport and slaughter.

2.2. Second Case

On 2 August 2021, *Salmonella enteritidis* positivity was recorded within a laying hen farm containing 62,300 ISA brown hybrids weighing about 1.880 kg at 32 weeks of age. After confirmation of infection by the National Reference Health Laboratory, animal health measures were implemented. In this case, as the hens were too young to have reached the ideal weight (which is about 2 kg for ISA Brown laying hens) corresponding to the production specifications adopted by the slaughterhouse, the farmer was forced to depopulate on the farm premises. Therefore, with the help of a specialist company, the animals were first caught in plastic bins and walked to the mobile CO_2 container (Figures 1 and 2).

Before injecting CO_2, hens were inserted into the device gradually (10/15 at a time) over the course of several minutes until they reached a total of 120–150 birds. At the end of operations, the depopulation cost to the farmer was 30,200 euros, with an average cost of 0.48 Euros per hen culled. The high price derives mainly from fixed costs such as the purchase/delivery of the CO_2 and the transport of the container to the farm.

Figure 1. Container where birds are placed before treatment with CO_2. On the left is the metal container where the chickens are placed, on the right is the CO_2 cylinder connected to the container.

Figure 2. Placement of birds into the container. After being caught and put into plastic bins, the hens were inserted through openings at the top of the container into which CO_2 was injected.

2.3. Third Case

On 22 July 2020, *Salmonella enteritidis* positivity was recorded within a laying hen farm containing 2500 ISA brown hybrids weighing about 2.5 kg at 70 weeks of age. No slaughterhouse accepted the positive batch because of the low profitability of a batch made of a such small number of birds and the too expensive cleaning and disinfection costs of the establishment. Moreover, applying the depopulation method using CO_2 containers would have been too expensive for a low number of infected animals.

Therefore, the farmer had to cull the birds directly on the farm via individual euthanasia with the drug T-61 under the oversight of a veterinarian. A culling point was set up on the farm, with nine people carrying out the different operations (such as capture of birds and carcass disposal) plus two veterinarians conducting euthanasia in two separate areas of the farm.

The birds were individually captured and transported to the veterinarian for euthanasia. Subsequently, the carcasses were placed in storage containers located on the farm itself. At the end of operations, the depopulation cost to the farmer was 3250 euros, with an average cost of 1.30 Euros per hen culled.

3. Results and Discussion

As shown in the three cases, when the presence of *Salmonella enteritidis* was confirmed on the farm, the following animal health measures were applied: (i) no eggs or poultry may leave infected holdings. However, by permission of the prefect, the owner may send the eggs to an approved establishment for the production of egg products in order to subject them to heat treatment for the destruction of *Salmonella* spp. before they are placed on the market. Eggs circulating through this authorization are considered category B eggs in accordance with Regulation (EC) No. 589/2008 of 23 June 2008; (ii) sampling for epidemiological investigations; (iii) elimination of infected bird flocks; (v) destruction of feed stored on the holding and distributed to the infected flocks; (vi) cleaning and disinfection of premises, surrounding areas, access routes, and vehicles used for the transport of poultry, followed by sanitary break [20,21].

In general, the elimination phase is crucial and not always easy. In fact, as evidenced by the first case report, the use of slaughter is very hard to practice due to the difficulty of a slaughterhouse accepting the batch of infected laying hens. Therefore, as a result of this difficulty, depopulation methods must be employed on the farm [15,18].

According to the AVMA's (American Veterinarian Medical Association) guidelines for depopulation of animals, the most commonly used methods to carry out this practice are based on atmospheric modification (insufflation of carbon dioxide, carbon monoxide, argon, or nitrogen in large containers); physical methods (cervical dislocation, bolting, or decapitation); or intravenous injection of euthanizing drugs [24].

As showed by the three case reports, the choice of method to be applied for the management of *Salmonella* spp. outbreaks depended heavily on the availability of the slaughterhouse (only in the first case report was there the possibility of using this practice), the number of birds on the farm, and their age.

Based on these conditions, in the second case report, the age and numerosity of the birds led the farmer towards the choice of employing mobile CO_2 containers for depopulation; while, in the third case report, the low number of birds led toward the choice of employing intravenously injected drug T-61 for individual elimination of infected laying hens.

Therefore, below we consider various factors relating to the method used. Firstly, it is important to start from the assumption that slaughtering is a beneficial practice for the elimination of infected bird flocks, because: (i) it allows better compliance with animal protection conditions because the birds are slaughtered in EU-authorized establishments in accordance with European Regulation No. 1099/2009 [22]; and (ii) it allows carcasses to be reused according to the measures set out in Regulation 1086/2011 [25].

However, as shown in the first case report, the main difficulty in implementing this method is finding a slaughterhouse near the farm that will accept a batch of infected animals, mainly because of the difficulties associated with cleaning and disinfection after slaughter [26]. This observation is in agreement with Marin et al. [27] that showed how the same strain of *Salmonella enteritidis* is able to survive common cleaning and disinfection protocols, generating cross-contamination between different slaughter stages. This is likely due to the pathogen's ability to form a biofilm that allows the bacteria to withstand disinfectant treatments, mechanical removal, and other types of stress much better [27].

Taking this into account, the farmer is forced to contact slaughterhouses located far away from the farm, and finding a service that can take the animals hundreds of kilometers away is an additional complication.

In addition, it is important to emphasize how stressful a long journey can be and that it could lead to the spread of *Salmonella* spp. among the animals [28,29]. Stress due to a long journey can generate a disruption of the intestinal microbiota and intestinal epithelium with a reduction in innate protective mechanisms, increasing the ability of *Salmonella* spp. to bind and colonize gut cells [28].

As shown in the second case report, where a farm has many birds, a specialist service can be brought in to eliminate infected animals using a mobile CO_2 container. Euthanasia by CO_2 is a practice that requires an increase in CO_2 levels in the environment (in this case the mobile container), resulting in a decrease in O_2. Increased CO_2 causes hypercapnia in the animal resulting in acidification of the blood and cerebrospinal fluid of the bird. Acidification of cerebrospinal fluid causes an increase in the rate of respiration with a further lowering of blood pH and consequent respiratory depression. In addition, acidification of cerebrospinal fluid also leads to acidification of brain cells that contributes to reduction of consciousness and brain activity. Therefore, reduced brain and respiratory functions and consequent hypoxia will lead towards total loss of brain, heart, and respiratory functions, followed by death [30].

Although this is a convenient way of culling many birds at a time, it involves major animal welfare problems, ranging from the capture to the culling phases.

Firstly, when using mobile equipment such as gas containers, animals need to be caught and walked to the culling device.

Capture can be extremely stressful for the animals and at the same time can lead to physical damage such as broken bones and bruising [31]. Before the culling stage, animals must be put into the mobile CO_2 container as quickly as possible to ensure that the first birds do not suffocate to death from the weight of subsequent animals [23,32].

Then, during the culling phase, CO_2 concentrations need to be monitored; indeed, CO_2 concentrations of above 40% generate distress and pain in humans, and it has been shown that the same concentration in animals can cause similar adverse feelings [33]. This happens because respiratory depression during CO_2 inhalation causes dyspnea, or difficulty in breathing. Several studies have reported how this sensation is distressing for birds [34,35]. In addition, CO_2 is an acidic gas that, when in contact with water on the mucous membrane, forms carbonic acid, causing pain [34,36]. Such issues could lead the farmer to being sued by animal welfare associations. Another disadvantage lies in the cost; in fact, compared to slaughtering, which provides an economic value to animal carcasses (placed on the market after heat treatment and microbiological controls carried out in the slaughterhouse), the CO_2 method has fixed costs linked to the purchase/delivery of the gas used and the transport of the containers, and does not allow for any recovery of the carcasses [25]. For these reasons, the method is not suitable for application to small farms with a low number of birds.

Few companies in the country can provide this service, so in case of multiple salmonellosis outbreaks on farms, these firms would not be able to provide an efficient service.

Therefore, in order to improve this problem, large-scale depopulation methods are currently being investigated such as "whole barn depopulation with carbon dioxide (CO_2)", in which a barn is sealed hermetically, and tons of CO_2 are injected inside it [36].

This method is quicker than using a mobile CO_2 container, as it allows all the birds in the barn to be killed at the same time and also has the advantages of not requiring animal capture, and of culling the animals within an environment that is familiar to them [36].

However, the risk of gas leaks that could pollute the surrounding environment, and at the same time, could delay animal deaths, limits its applicability [23]. In addition, this method is also not very cost-effective as it requires large numbers of personnel and high quantities of gas [36]. Therefore, if this method is to be an effective solution to disasters such as multiple salmonellosis outbreaks, further studies and improvements to it will be needed. Finally, as shown in the third case report, in the event of salmonellosis on farms with small numbers of birds, where slaughter is not possible, individual euthanasia of the animals can be performed by injecting T-61, a nonbarbiturate, non-narcotic combination of embutramide, mebenzonium iodide, and tetracaine hydrochloride [37]. The anesthetic and paralytic action of the drug are capable of depressing brain activity, inhibiting consciousness, and the respiratory control centers of the brain, thus inducing respiratory and circulatory collapse [38]. Since this method involves injecting the drug into individual animals, it requires licensed veterinarians to perform both the euthanasia and monitoring of the animals [23], and a sufficient number of personnel to capture the animals and properly dispose of the carcasses, which, according to European Regulation No. 1069/2009, will be treated as category 2 animal by-products [39].

In addition, carcasses have to be disposed of properly because if ingested by animals, secondary toxicity may occur [38], except for when the farm adheres to certifications that entitle to fair compensation according to European Regulation No. 690/2021 and European Regulation (EU) No. 429/2016 [15]. Therefore, T-61 euthanasia is also a drawback for the farmer who has to pay for carcass disposal. One critical aspect of this method is that animals have to be captured individually before receiving the drug, which raises both the risk to operators and the stress felt by the animals [31]. This makes it advisable to couple the use of T-61 with an anesthetic substance, such as alpha chloralose, administered in feed, to make the catching phase easier.

Interest in alpha chloralose dates back to 1972, when Lees et al. described its scientific basis [40]. Loibl et al. [41] showed that in domestic chickens, its anesthetic effect occurs when the effective dose (ED50) is 45 mg/kg, while the lethal dose (LD50) requires a concentration of 300 mg/kg [41]. In the United States, alpha chloralose is utilized by the USDA wildlife services as an immobilizing agent to capture wild birds, there being a special authorization from the Food and Drug Administration, known as Investigational New Animal Drug (INAD) [42].

As well as in the United States, this substance is also used in New Zealand to control wild bird populations [43].

In Europe since 2009, alpha chloralose (Product-Type 14) has been included as a biocide active substance according to "Commission Directive 2009/93/EC of 31 July 2009 amending Directive 98/8/EC of the European Parliament and of the Council to include alphachloralose as an active substance in Annex I thereto" [44].

Pursuant to Article 86 of European Regulation No. 528/2012 "concerning the making available on the market and use of biocidal products" [45], Decision (EU) 2021/333 of 24 February 2021 postpones the expiry date of approval of alpha chloralose (Product-Type 14) to 31 December 2023 [46]. In addition, as of 2007, chloralose is no longer approved as an active molecule for pesticide production in Europe [47]. As a result, due to this European decision, there is currently no veterinary medicine that incorporates alpha chloralose as an active ingredient. Therefore, in order to improve biosafety conditions during depopulation operations using T-61, the reasons for the withdrawal of alpha chloralose from the market should be investigated, while the National Veterinary Drug Agency should be asked to authorize its production and sale as an oral anesthetic in birds.

Thus, *Salmonella* spp. infections on laying hen farms are a recurrent health risk, requiring heightened awareness on the part of the farmer when establishing the health control plan and more detailed analysis by the competent authority in managing the depopulation site in the event of positivity. The critical points related to the three methods discussed are summarized below (Table 1).

Table 1. Critical points related to slaughtering, euthanasia by CO_2 and euthanasia by injection of T-61.

Slaughtering	Euthanasia by CO_2 in Farms with a Large Number of Birds	Euthanasia by Injection of T-61 in Farms with Few Birds
A slaughterhouse located near the farm that can accept the *Salmonella* spp.-positive batch may be hard to find	High costs	High costs associated with carcass disposal
Stress suffered by animals on very long journeys.	Compliance with animal welfare rules may be an issue	Compliance with animal welfare rules (due to stress during the capture phase) may be an issue
A transporter to take the batch to slaughterhouses located not too far away from the farm may be hard to find	A specialist company in the event of multiple salmonellosis outbreaks in the country may be hard to be find	No carcass enhancement

4. Conclusions

The application in the field of the requirements contained in Reg. (EU) 429/2016 and Reg. (EC) 2160/2003, in the case of positivity for *Salmonella* spp. in laying hen farms, revealed critical management issues.

In the first case, we experienced difficulties in resorting to slaughter although it appears to be the method that most protects animal welfare. In the second case, euthanasia by CO_2, although it is an alternative to slaughter in the case of farms with many birds, shows several critical aspects concerning animal welfare [22].

Among euthanasia methods that employ CO_2, an interesting approach that shows research potential for future studies is probably the "whole barn" approach.

Similarly, in the third case, it was pointed out that the use of T-61, preferably used in small farms, requires more studies from the anesthetic perspective, especially following the withdrawal of alpha chloralose. Therefore, in order to avoid unnecessary animal suffering and distress, new studies are needed to encourage ministerial veterinary departments to authorize alpha chloralose as an oral anesthetic drug in birds. In addition, to prevent such situations from occurring, management strategies and preventive-medicine programs have to be adopted (i) to improve biosecurity regulations both external and internal on farms; (ii) to optimize the environmental conditions of the sheds (ventilation, litter, humidity, stocking density, lighting); (iii) to use natural agents (probiotics, prebiotics, enzymes, organic acids, immunostimulants, essential oils, etc.) instead of antibiotics, and (iv) to carry out vaccination against salmonellosis on poultry [48–50]. A management strategy of poultry farms guided by animal welfare could guarantee the production of healthier and more resistant animals, with a significant reduction of the use of antibiotics while preserving environment and consumer health [48,50].

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