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Risk factors for *Salmonella enterica* subsp. *enterica* persistence in broiler-chicken flocks on Reunion Island

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ABSTRACT

This study was conducted to identify the main risk factors for *Salmonella* spp. persistence in broiler flocks in Reunion Island. Seventy broiler farms were surveyed from March 2016 to June 2018. Samples of fresh droppings were collected using gauze socks, and a questionnaire was completed with the farmers. Persistence was defined as an infection with the same serovar before and after cleaning and disinfection (C/D) of poultry houses. *Salmonella* spp. was found to persist on 27% of the farms. Cleaning concrete surrounding areas (OR = 0.23) and disinfecting silos (OR = 0.17) reduced the risk of pathogen persistence. An analysis of infections of pests found in the vicinity of the farms confirmed their role in the persistence of *Salmonella* spp. Fifteen percent of the pests were infected and the presence of mealworms in poultry litter (OR = 6.69) was found to increase the risk of *Salmonella* spp. persistence. We conclude that improved cleaning-disinfection, sanitary preventive measures and pest control in the poultry sector are needed to avoid the persistence of *Salmonella* spp. on broiler farms.

1. Introduction

Non-typhoidal *Salmonella* spp. remains a public health burden worldwide, causing 1.3 billion cases of gastroenteritis and three million deaths per year (Bhunia, 2008). *Salmonella* spp. is the second leading zoonotic disease agent in the European Union, with 88,715 cases reported in 2014 (EFSA, 2015) and *Salmonella* spp. is the leading cause of bacterial food-borne diseases.

The foods most commonly implicated in outbreaks of human salmonellosis are of animal origin, including contaminated eggs and poultry meat (Van Immerseel et al., 2005).

Reunion Island is a French overseas tropical territory located in the Indian Ocean. Although only 8,700 tons of chicken meat are produced each year, it is the main source of animal protein (40 kg/inhabitant/year) and 25% of chicken is consumed in the form of processed products (notably sausages) (Trimoulinard et al., 2017). Reunion Island has been already hit by *Salmonella* spp. (Henry et al., 2012; D'Ortenzio et al., 2008) and *Salmonella* spp. have been shown to cause 22.2% of food-borne infections between 1996 and 2005 (D'Ortenzio et al., 2008).

To prevent contamination of chicken carcasses, infection by *Salmonella* spp. needs to be controlled all along the food production chain (Mead, 1993). On-farm rearing conditions are considered to be a key point in controlling *Salmonella* spp. (Bailey et al., 2001). The most recent publications identified risks of horizontal transmission of *Salmonella* spp. on broiler farms. These risks include inadequate cleaning and disinfection of broiler rearing houses which lead to contamination of the following flock (Lahellec C et al., 1986; Davies and Wray, 1996; Higgins R et al., 1981; Rose N et al., 1999; Rose N et al., 2000), poor levels of hygiene (Henken et al., 1992), contamination of feed (Davies et al., 1997) and the presence of mealworms in the chicken house and of rodents on the farm (Baggesen et al., 1992; Löhren, 1994). Recent research showed that peri-domestic fauna including rats, shrews, cockroaches and birds are carriers of *Salmonella* spp. on Reunion Island (Tessier et al., 2016).

One of the main problems which affect broiler farms is the persistence of the same *Salmonella* spp. serovar between two consecutive flocks, and the fact that very little information is available to understand such persistence on Reunion Island.

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The aim of the present study was therefore to evaluate the rate of *Salmonella* spp. persistence in broiler houses on the island and to explain the factors that are associated with persistence.

2. Material and methods

2.1. Study sample

Our study included 70 broiler farms (out of a total of 180 on the island), and was carried out between March 2016 and June 2018. The location of the farms and the chick placing day were provided by veterinary practitioners. Farm selection was initially random but only owners who were willing to cooperate were included in the study.

2.2. Sample collection

Each farm was visited four times and the samples were taken using pairs of sterile gauze socks made of absorbent woven cotton (Sodibox, Nevez, France) to allow large areas to be easily surveyed. Each pair was used for 50% of the surface, particular attention was paid to passages through freshly soiled areas and/or areas that receive high densities of animals. On the first visit, samples of fresh droppings were collected from the previous flock just before slaughter and gauze socks were used to swab the walls. On the second visit, several gauze socks and swabs were used on the floor and on the walls of the poultry house, in the access lock and outdoors near the poultry house to check the effectiveness of cleaning-disinfection. The farm was visited again just after day-old chicks had been delivered. The final visit took place at the end of the rearing period just before slaughter and gauze socks and swabs were used on the walls and on the floor to evaluate the *Salmonella* spp. status of the current flock.

Shrews, mice, rats, flies, ants, cockroaches and birds were also caught near the poultry houses using sticky traps. Live cockroaches, ants, flies and mealworms were transported to the laboratory and immersed in 90% ethanol to decontaminate their outer surface before being air dried and crushed. The intestines and liver of shrews, rats and mice were aseptically removed and cloacal swabs were taken from birds.

A questionnaire addressing factors that could contribute to the persistence of *Salmonella* spp. (poultry environment, farm staff and visitors, poultry house, cleaning and disinfection and pests) was filled in with the farmers. The questionnaire was pre-tested on three farms and was subsequently always presented by the same team who had been specifically trained for this purpose.

2.3. Ethics statement

All the animal procedures carried out in this study were performed in accordance with European Union legislation for the protection of animals used for scientific purposes (Directive, 2010/63/EU). The ethical terms of the research protocol were approved by the CYROI Institutional Review Board (*Comité d'Ethique* du CYROI n° 144).

2.4. Salmonella spp. isolation and identification

Salmonella spp. detection was adapted from EN ISO 6579/A1: 2007. Each sample was pre-enriched in buffered peptone water (BPW; BioRad, California, USA) and incubated at 37 °C for 18 h ± 2 h. A 1mL aliquot of the pre-enrichment broth was used to inoculate 10 mL of Müller–Kauffmann tetrathionate broth (MKTTn; BioRad, California, USA) and 0.1 mL was used to inoculate Modified Semi-solid Rappaport Vassiliadis agar plates (MSRV; BioRad, California, USA). The two media were incubated at 37 °C for 24 h and at 41.5 °C for 24–48 h, respectively. From a migration zone on MSRV ≥20mm and from the MKTTn broth, plating was accomplished by streaking the cultures on *Salmonella*-Shigella (SS) agar plates and xylose lysine deoxycholate (XLD) agar plates (BioRad, California, USA). The inoculated plates were incubated at 37 °C for 18–24 h. After incubation, whenever possible, four typical *Salmonella* spp. colonies per sample were purified and biochemically identified by assays on Kligler-Hajna medium (BioRad, California, USA), Mannitol Motility Test Medium (BioRad, California, USA), Urea-indole broth (BioRad, California, USA) and an o-nitrophenyl-β-D-galactopyranose (ONPG) disk (BioRad, California, USA). Biochemically confirmed colonies were then serotyped according to the Kauffmann-White scheme and using a slide agglutination test with *Salmonella* spp. polyvalent O and H antisera (Diagnostic Pasteur, Paris, France).

2.5. Definition of outcome variable

The observation unit was the flock. Persistence was defined as poultry infection with the same *Salmonella* spp. serovar in two consecutive flocks. The outcome variable was thus dichotomous.

2.6. Definition of explanatory variables

Fifty-six closed questions were addressed in the questionnaire and all variables were categorical. The number of categories per variable was limited, so that the frequencies of categories were >10%. All bivariate relationships between explanatory variables were checked ($\chi 2$). For bilateral relationships with strong statistical associations and biological plausibility, the one most related to the outcome variable was chosen.

2.7. Statistical procedure

A two-stage procedure was used to assess the relationship between explanatory variables and *Salmonella* spp. persistence. Logistic regression was used according to the method described by Hosmer and Lemeshow (2000). In the first stage, a univariable analysis was performed to link *Salmonella* spp. persistence to each variable. Only factors associated (Pearson $\chi 2$ test, $P < 0.20$) with *Salmonella* spp. persistence were included in a full model in R software for multivariable analysis (Table 1) (Mickey and Greenlands, 1989). The second stage involved a logistic multiple-regression model. The contribution of each factor to the model was tested with a likelihood-ratio $\chi 2$ through a stepwise backwards and forwards procedure. At the same time, the simpler models were compared to the full model using the Akaike information criterion (Akaike, 1974). This process was continued automatically until a model was obtained with all factors significant at $P < 0.10$ (two-sided). The goodness-of-fit of the final model was assessed using Pearson $\chi 2$, deviance and the Hosmer–Lemeshow tests (Hosmer and Lemeshow, 2000).). Interactions were not tested (because of the small sample size).

3. Results

In our study, a total of 1,120 samples were collected (16 samples per farm). The persistence of *Salmonella* spp. from one flock to another was associated with the *Salmonella* spp. status of the previous flock and cleaning-disinfection of the premises (Table 2).

The persistence level of the same serovar of *Salmonella* spp. before and after cleaning-disinfection of the poultry house was 27% (19 out of 70; 95% CI = [16.73; 37.56]).

The presence of pests was also associated with the persistence of *Salmonella* spp. on the farms. Fifteen percent of the 102 pests captured (15/102) were found to be infected by *Salmonella* spp. the main contaminated species were shrews, rats and flies (Table 3).

The persistence of *Salmonella* spp. from one flock to another was associated with the poultry house environment and cleaning-disinfection of the outdoor area near the chicken house. Out of the 56 variables tested in the screening analysis, three were used in the final model (Table 4). A

Table 1. Definition of explanatory variables adopted after univariable analysis for the logistic model ($p < 0.2$) included in the analysis of *Salmonella* spp. persistence in 70 broiler flocks on Reunion Island.

Variable	Level	[a] r/n	p-value
Poultry environment			
Altitude	<200 m	8/16	0.0675
	200 ≤ metres <300	2/7	
	≥300 metres	9/47	
Restricted access to the poultry houses	Yes with a fence	10/32	0.1138
	Yes with a chain	1/14	
	No restriction	8/24	
Special clothing provided for staff	Yes	8/21	0.1851
	No	11/49	
Poultry house			
Age of poultry house	New ≤12 years old	5/28	0.1463
	Old >12 years old	14/42	
Type of poultry house	Louisiane	5/29	0.0440
	Colorado	11/24	
	Péi "locally made"	3/17	
Type of ventilation	Static	0/19	0.0001
	Dynamic	19/51	
Adequate functioning footbath	Yes	6/31	0.1866
	No	13/39	
Cleaning and disinfection			
Surface cleaning of fans	Yes	16/39	0.0022
	No	3/31	
Complete cleaning of fans (skirts)	Yes	13/27	0.0055
	No	3/28	
	Not available	3/15	
Equipment disassembled for cleaning	Yes	16/47	0.0168
	No	3/23	
Cleaning of the equipment	Yes	18/54	0.0176
	No	1/16	
Cleaning of silos at the end of the rearing period	Yes	9/42	0.1908
	No	10/28	
Cleaning of surrounding concrete area	Yes	12/57	0.0221
	No	7/13	
Decontamination of concrete outdoors strips	Yes	6/34	0.0795
	No	13/36	
Disinfection of silos at the end of the rearing period	Yes	10/53	0.0081
	No	9/17	
Freezer to store dead animals	Yes	3/19	0.176
	No	16/51	
Pests			
Presence of shrews	None	6/13	0.0171
	few ≤2	11/33	
	many >2	2/24	
Presence of mealworms	Yes	16/38	0.0013
	No	3/32	
March 2016–June 2018			

[a] r = number of flocks with persistent *infection by* ***Salmonella* spp.** and n = total number of flocks per variable.

decreasing risk of persistence in the flock was associated with cleaning the surrounding concrete area and disinfecting silos. The risk of persistence increased when mealworms were also found to infest the premises.

4. Discussion

Our study confirmed the high level of persistence of *Salmonella* spp. after cleaning-disinfection in farms in Reunion Island (the same serovar was found to persist in 27% of the farms investigated).

To evaluate persistence, we considered the serovar of the strains, but for more certainty we should have compared the genotype of each strain. We checked the hatchery (there is only one chicken hatchery on the island) as a potential source of infection with the same serovar and confirmed that no infection occurred at their level during the course of our study. We also believe that the fact the farmers had to be willing to cooperate during the lifespan of the flock may have led to a selection bias. Nevertheless, sampling was representative of the location of the farms on the island. The limited duration of the study and the fact that data were only collected by two people trained specifically for the purpose certainly contributed to the repeatability of the results and we used the international reference method for detecting *Salmonella* spp. for the bacteriological analysis (EN ISO 6579/A1: 2007).

Table 2. *Salmonella* spp. infection of poultry flocks after cleaning-disinfection according to *Salmonella* spp. infection of the previous flock (70 flocks, Reunion Island, 2016–2018).

Serovar	Still infected by *Salmonella* spp. after cleaning-disinfection								
	Livingstone	Newport	Typhimurium	Weltevreden	Agona	Virchow	Montevideo	Negative	Total
Infection of the previous flock by *Salmonella* spp.*									
Livingstone	**7**							3	**10**
Newport		**3**							**3**
Typhimurium			**4**					3	**7**
Weltevreden									**0**
Agona					**1**				**1**
Virchow						**2**			**2**
Livingstone + Typhimurium					1				**1**
Montevideo							**2**		**2**
Negative	1			1				42	**44**

Numbers in bold show the Salmonella serovar present in the previous flock and still present after cleaning and disinfection.
* (before cleaning-disinfection).

Table 3. *Salmonella* spp. infection of pests (70 flocks, Reunion Island, 2016–2018).

Pests infected	Mealworms	Shrews	Rats	Flies	Ants	Mice	Cockroaches	Birds	Total
Serovar									
Livingstone	1								**1**
Newport	1	1	2						**4**
Typhimurium		2		2					**4**
Weltevreden		3		1	1				**5**
Enteritidis				1					**1**
Negative	19	16	4	22	8	3	8	7	**87**
Total	**21**	**22**	**6**	**26**	**9**	**3**	**8**	**7**	102

Numbers in bold show the total for each modality.

Our study showed the limitations of current C/D procedures applied on farms in Reunion Island, which were not strict enough to effectively eliminate *Salmonella* spp. Although the health status of the previous flock has already been mentioned as a risk factor in a number of studies in tropical regions (Cardinale et al., 2004a, b) and temperate regions (Marin et al., 2011; Namata et al., 2009), the C/D stage is essential to avoid the persistence of *Salmonella* spp. in two consecutive flocks. This step requires removing all organic and inorganic debris from surfaces capable of harbouring microorganisms and that may reduce the efficiency of disinfection (Cardinale et al., 2004a, b). Using specialized tools such as a high-pressure cleaner or a foam gun effectively eliminates organic matter (Davies and Wray, 1996; Moretro et al., 2009). Most of the poultry farmers on Reunion Island own a high-pressure cleaner, but the questionnaires showed that the flow and the pressure of the device were often below the recommended thresholds. In addition to a detergent for cleaning, disinfectants are also required to eliminate *Salmonella spp*. The farmers on Reunion Island do not respect the appropriate doses of disinfectants thus probably preventing efficient decontamination.

In an environment in which the density of poultry houses is increasing, the application of strict sanitary and hygiene measures by poultry farmers is essential to limit the entry and spread of pathogens. Pests on farms can act as mechanical or biological vectors of *Salmonella* spp. Many studies have shown pests play a major role in the epidemiology of *Salmonella* spp. on farms (Davies and Wray 1995; Kinde et al., 1996; Rose et al., 2000; Davies et al., 2001; Davies and Breslin, 2003; Gradel and Rattenborg, 2003).

In our study, *Salmonella* spp. was isolated from 15% of the pests collected around the farms surveyed. These results are in agreement with those of other studies on Reunion Island (Tessier et al., 2016) and in Spain (Marin et al., 2011) where the prevalences were 12% and 14%,

Table 4. Risk factors for *Salmonella* spp. persistence in broiler-chicken flocks (70 flocks, 20162018).

Variables	*Salmonella* spp. persistence in the flock as a function of the variable (%)	Logistic regression model		
		OR	IC 95%	p-value
Cleaning of concrete surrounding area				
Yes	21	0.23	0.06–0.93	0.036
No	54	-		
Disinfection of silo				
Yes	19	0.17	0.04–0.63	0.007
No	53	-		
Presence of mealworms				
Yes	42	6.69	1.48–30.34	0.006
No	9	-		

Intercept = 0.08673, model deviance = 61.249, AIC = 69.249, df = 5.

respectively. In our study, 45% of *Salmonella* spp. -persistent farms also hosted infected pests in the vicinity of the farms. Of these pests, rats and shrews were the most infected, with prevalences of 33% and 27%, respectively. The infection rate found in Spain was 5.4% in rodents (Marin et al., 2011), and in Reunion Island, it was 7% in rats and 22% in shrews (Tessier et al., 2016). These pests are thus a direct source of contamination for poultry, either after consumption of infected wildlife, or indirectly after contact with infected faeces (Meerburg et al., 2006). A study by Wales et al. (2006) underlined the ability of vectors including rodents to amplify the concentration of *Salmonella* spp. present in the environment. These pests are abundant on farms on Reunion Island because of their proximity to sugar cane fields (Henry, 2011). After the harvest, rodent populations migrate preferentially to poultry houses to find food (Henry, 2011).

Insects represented another way for *Salmonella* spp. to persist on farms. The prevalence of flies and mealworms was 15% and 9.5%, respectively. Levels of *Salmonella* spp. infection have been found to vary flies: 13.6% in Spain (Marin et al., 2011), 67% in Burkina Faso (Barro et al., 2006) and 14% in Malaysia (Choo et al., 2011). In their study, Pava-Ripoll et al. (2012) showed that pathogenic bacteria multiplied in the intestinal tract of flies and were three times more abundant in the gut than on the surface of fly bodies. Flies can thus be considered as biological vectors of *Salmonella* spp., contaminating their environment both through regurgitation and defecation. However, we did not catch enough mice, ants, cockroaches, and birds to conclude on the prevalence of *Salmonella* spp. in these pests.

Our statistical analysis also revealed the presence of mealworms in poultry houses to be associated with increased risk of the persistence of *Salmonella* spp. on farms (OR = 6.69). Roche et al. (2009) showed that ingestion of infected larvae by broilers or of lesser mealworms by adults is one possible route for *Salmonella* spp. transmission. Mealworms also act as mechanical vectors of *Salmonella* spp.. Crippen et al. (2012) demonstrated that after only 2 h of exposure, lesser mealworms could acquire the environmental bacterium and disseminate it in the poultry house for an average of eight days. Under our conditions, mealworms were observed regularly in broiler houses, even during C/D, they survived in the cracks in the building floor and walls and are then probably able to harbour *Salmonella* spp. and spread them to the following flock. Skov et al. (2004) showed that the occurrence of *Salmonella* spp. in two consecutive broiler flocks coincided with the presence of mealworms infected with the same serovar when the chicken houses were empty.

A second risk factor identified was the lack of cleanliness of concrete areas around the chicken houses (OR = 0.23). Outdoor areas can be soiled by the movements of vehicles and people entering and leaving the farm, such as exporting poultry, removing manure removal, delivering chicks, installing litter or delivering feed. Thus, non-compliance with biosecurity measures may lead to cross-contamination from the outside environment to the inner poultry house. In addition, pests are in direct contact with outdoor areas, as reported in 2004 by Jensen et al. (2004) and Rodenburg et al. (2004) To avoid attracting pests, outside areas should be kept clear of all bulky waste. Concreting these areas is also recommended to facilitate cleaning and to ensure more effective disinfection. Cardinale et al. (2004a, b) showed that thorough cleaning and disinfection of the area surrounding the poultry houses, and disposing manure outside the farm, were associated with reduced risk of *Campylobacter* spp infecting the flock.

Finally, non-disinfection of feed silos was the third risk factor we identified (OR = 0.17). Feed can be contaminated upstream of the silo, either directly at the production plant or, for example, by birds depositing droppings in the delivery trucks. Heyndrickx et al. (2002) reported that feed in poultry houses is a risk factor significantly linked with flock status. Furthermore, as observed during our visits to farms, pests such as mealworms can successfully enter silos and deposit contaminated droppings directly into feeding systems when feed is being stored in the silo, during opening, or when storage is defective. When the silos are not disinfected, the temperature and humidity and the presence of organic matter will facilitate bacterial multiplication.

To sum up, three risk factors for the persistence of *Salmonella* spp. in the flock were identified. The *Salmonella* spp. status of the previous flock, the effectiveness of cleaning-disinfection of the poultry house as well as of the surrounding outside area, and the presence of pests contribute to the persistence of *Salmonella* spp. Most of these risks are already reported in the literature, but this is the first time that such results concern a tropical island. The environmental pressure linked to the hot and humid tropical climate, and the high density of farms and agricultural activities calls for more stringent hygiene and biosecurity protocols in broiler farms on Reunion Island.

Declarations

Author contribution statement

Ethèves M.A., Cardinale E.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Choisis N.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Alvarez S.: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Dalleau F., Hascoat J.: Performed the experiments.

Gallard V.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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