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Salmonella contamination of laying-hen flocks in two regions of Algeria

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ABSTRACT

A preliminary epidemiological study of *Salmonella* contamination in laying-hen flocks was carried out in the regions of Annaba and Eltarf, Algeria, from March to October 2008 and March to November 2009. Our objectives were (i) to estimate the prevalence of infection by *Salmonella* spp. in seven pooled samples during the hens' laying period (ii) to identify the serotypes and antimicrobial resistance phenotypes of isolates, and (iii) to characterize the factors that may be related to *Salmonella* contamination in Algerian henhouses. For this purpose, 18 out of 22 operational laying-hen houses were sampled one to three times during these periods: once at the start of laying (pullets aged 22–31 weeks), once in the middle of laying (47–60 week) and once at the end of laying prior to depopulation (70–86 week). The flocks'*Salmonella* status was assessed by collecting 2754 environmental samples that were analyzed according to the ISO 6579 method. The antibiotic resistance of *Salmonella* strains was tested as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The relationship between each potential risk factor and the *Salmonella* status of laying-hen flocks was evaluated by calculating the relative risk with 95% confidence intervals. Eight flocks tested positive for *Salmonella* spp., with a higher prevalence at the end of laying than at either the beginning or middle. Only 19 isolates were recovered from the 2754 samples analyzed and nine different serotypes identified. *S.* enteritidis (n = 4) was the most prevalent serovar, along with *S.* Kentucky and *S.* Hadar (n = 3), followed by *S.* Heidelberg, *S.* Manhattan and *S.* Virchow (n = 2), whereas *S.* Dublin, *S.* Typhimurium and *S.* Albany were found only once. Thirteen isolates were resistant to at least one antimicrobial agent. Of these, six were resistant to at least three different antimicrobial classes. *Salmonella* serovar Kentucky isolates were resistant to fluoroquinolones with ciprofloxacin MIC ≥ 8 mg/L. Six risk indicators were identified as potentially related to the *Salmonella* status of layer houses.

1. Introduction

Non-typhoid *Salmonella* is a major bacterial pathogen and the second cause of food-borne diseases in Europe and worldwide (Bouwknegt et al., 2004; Collard et al., 2007; EFSA, 2007a). In France, *Salmonella* was isolated from 64% of food-borne outbreaks between 1996 and 2005. These *Salmonella* outbreaks were the cause of 49% of all reported cases (Delmas et al., 2006). In the United States, the Centers of Disease Control and Prevention (CDC) estimate that approximately 40,000 cases of human salmonellosis were diagnosed annually in the 1990s, and that the annual number of non-typhoidal salmonellosis cases was about 1.4 million (Voetsch et al., 2004). In addition to the health consequences, human *Salmonella* infections have a significant financial impact. A study in the Netherlands estimated the costs of *Salmonella* infections in 1999 at € 4 million (Van den Brandhof, de Wit, de Wit, & Van Duynhoven, 2004). An American study (Bryan & Doyle, 1995) estimated that the costs per human salmonellosis case range from approximately US $ 40 for uncomplicated cases to US$ 4.6 million for those ending with hospitalization and death. There are no accurate data on *Salmonella* prevalence in developing countries.

One of the most common sources of human gastroenteritis is the consumption of *Salmonella*-infected poultry products, especially eggs and poultry meat (EFSA, 2009). *S.* Enteritidis is, together with *S.* Typhimurium, the most commonly isolated serotype in human cases of salmonellosis in Europe (EFSA, 2007a; WHO, 2006), and contaminated eggs still remain the biggest source of infection from

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Salmonella Enteritidis for humans (De Jong & Ekdahl, 2006; Gillepsie et al., 2005).

The combination of stringent hygiene measures and vaccination has considerably reduced vertical transmission of *Salmonella* infection from parent flocks (Van Immerseel et al., 2004) Horizontal transmission remains the major source of *Salmonella* contamination in broiler hen and egg productions (Chadfield, Permin, Nansen, & Bisgaard, 2001; Shirota, Katoh, Murase, & Otsuki, 2001). Only a few epidemiological studies have been carried out to investigate *Salmonella* infection risk factors in laying-hen farms. Various risk factors were identified in these studies, including (1) large flocks (Mollenhorst, Van Woudenbergh, Bokkers, & de Boer, 2005; Namata et al., 2008) (2) the age of the sampled hens, older hens being more likely to be contaminated by *Salmonella* (Castellan et al., 2004; Namata et al., 2008), and (3) inefficient cleaning and disinfection after depopulation for flock renewal (Davies & Breslin, 2003).

It is a common practice in modern food animal husbandry to use antimicrobials for both therapeutic and prophylactic purposes (Aarestrup, 2004; Berge, Atwill, & Sischo, 2003; EFSA, 2008). The subsequent emergence of antimicrobial resistance compromises animal health by causing therapeutic failure. Furthermore, there may be adverse consequences for public health because of the spread of antimicrobial resistance from animals to humans, leading to treatment failure in human infectious diseases (EFSA, 2009; Jordan et al., 2009).

In Algeria, poultry and egg productions have increased substantially since the mid-1980s, supported by an incentive policy. Poultry production has increased from 162,000 t in 1985 to 270,000 t in 1995, largely due to state subsidies. It currently provides 10 kg of poultry meat annually per capita, and fresh egg production is self-sufficient. According to Algerian agricultural ministry data, egg production increased from an average 32,000 t in 1982 to 120,000 t in 1995 (Anonymous, 2003). Egg consumption has also increased from 96 eggs annually per capita to 120 in 1989 (Anonymous, 2003).

Concomitantly, several outbreaks of salmonellosis have been observed in Algeria during recent years in both humans and food animal production, particularly poultry (Aboun et al., 2003; Ayachi, Alloui, Bennoune, & Kassah-Laouar, 2010). However, the public health consequences and economic impact of *Salmonella* in the Algerian poultry breeding sector are unknown because there are no epidemiological surveillance systems or monitoring programs for *Salmonella* infections in this country.

The main aims of this study were therefore (i) to determine the prevalence of *Salmonella* in laying-hen flocks in 2 regions of Algeria, (ii) identify the serotypes present in these regions and their antimicrobial resistance, and finally (iii) to evaluate the risk factors that might influence the *Salmonella* contamination of laying-hen flocks.

2. Material and methods

2.1. Study design

The study was conducted in two regions in the eastmost part of Algeria from March 2008 to November 2009. The list and location of farms were obtained from official veterinary authorities for these regions and through our epidemiological investigation. During these two years, 22 operational layer houses were studied in both regions. All henhouse owners were invited to participate in the study, with absolute anonymity guaranteed for the farmer. A positive result would not have any adverse consequences. In all, 18 out of 22 layer houses with only one laying flock participated in the study for both years. Two farm owners (representing four layer houses) did not wish to participate. Flock sizes ranged from 2400 to 7200 hens. Pullets were loaded when 18 weeks old. Thirteen flocks comprised Isa Brown hens and five comprised Hy-Line hens. No sign of disease or pathology was found on the farms. Started pullets were typically vaccinated against Newcastle, Marek and Gumboro diseases. None of the sampled flocks was vaccinated against *Salmonella*. In most cases, owners are not just farmers and consider poultry farming a secondary occupation. The exact number of farmers may therefore fluctuate each year depending on the egg market and feed price.

All the henhouses in these regions are fitted out with conventional battery cages. Most of the buildings (16/18) have natural ventilation. Six out of 18 buildings had mixed structures: solid brick and cement walls, with a roof of zinc sheets. Fourteen layer houses were connected to the public electricity network and only four had an electric generator. One farmer raises his own pullets, but all the other farmers in these regions buy started pullets from rearing farms either in the same regions (7/18) or in neighboring regions (10/18). Most farmers obtained feed from a single public company or from five private organizations in these regions. The productive lifespan of laying hens is around 15 months.

2.2. Time of sampling

Samples were taken in two successive years, from May to October 2008 and March to November 2009. Thirteen hen flocks were sampled three times, but layer house access difficulties resulted in two flocks being sampled twice and three only once. The first visit was scheduled at the beginning of the laying period (pullets aged 22–31 weeks) for 15 hen flocks, the second about halfway through laying (47–60 weeks) for 14 flocks, and the third visit was late in the laying period, prior to depopulation (maximum 9 weeks before) for 17 flocks. The age of hens in the last henhouse sampled ranged from 70 to 86 weeks. Four henhouses were sampled at the end of laying (74–82 week) for an initial flock and at the beginning and middle of the laying period in the subsequent flocks living in the same henhouses. A flock was considered *Salmonella*-positive when the bacterium was isolated from at least one sample in the flocks.

2.3. Questionnaire design

A questionnaire was filled in during an onsite interview while collecting samples. It was used to gather information on the general characteristics of laying-hen flocks, dead-bird management, control of rodents and other domestic animals, biosecurity measures, farm staff and visitors, vaccination and administration of antibiotics. Of the questionnaire's 95 questions, 75% were closed-ended. The visit was completed by filling in a questionnaire based both on personal observations and on information obtained from the employees in charge of looking after the laying-hen flocks. A supplemental Table includes the questionnaire's 95 questions.

2.4. Sample collection

A total of 2754 samples were collected over two periods. The following seven pooled samples were collected from each flock at each visit: two pools of 15 fresh feces samples from under the cages, one pool of five fecal samples from the cages themselves, one pool of seven surface swabs, one pool of seven dust swabs from inside and outside the cages, and two pools of feed and water samples. On each sampling visit, 61 samples were collected from henhouses containing 4800 to 7200 hens, and 57 samples from henhouses containing 2400 to 3100 hens.

Pools of 15 fresh feces samples were collected randomly throughout the henhouse from the ground under the cages. To ensure that the samples were representative, the building was divided into five sectors and three fresh feces were collected from each sector. Each pooled feces sample weighed approximately 150 g. Fecal samples were scraped off the floor under the cages. These samples weighed approximately 50 g.

One pool of seven samples was collected on swabs from cages, and 1 pool of seven surface samples was collected from different places in each henhouse, such as walls, water and feeding equipment.

For technical and economic reasons, during the first visit, we used cotton-tipped swabs for environmental samples. The cotton-tipped swab was moistened with sterile water and used to swab 5–10 cm of cage surfaces, beneath and inside empty cages or troughs under nipple lines. The cotton-tipped swabs were also used to wipe 20 cm² of feeding and watering equipment. For this, each layer house was divided into seven subsections, with three cotton-tipped swabs for each subsection.

During the second sampling period, four drag swabs (AES Chemunex, Combourg, France) were used to collect dust from seven cages in various places within each henhouse. Each swab was sufficiently absorbent to collect the dust and dry manure from the surfaces beneath and inside two empty cages per swab.

Three drag swabs were also used to swab a 0.5 m^2 surface area of feeding equipment and approximately 30 cm² per swab of henhouse walls. One pool of four samples of feed and one pool of four samples of water were collected from flocks with 2400 to 3100 hens. Four 5 g sub-samples of feed were collected from feeders, and four 5 mL sub-samples of water were collected from nipples and reservoirs. For flocks with 4800 to 7200 hens, six 5 g sub-samples of feed and six 5 mL sub-samples of water were collected from feeders, nipples or reservoirs.

Samples were taken from different places on each sampling visit. The pooled samples were placed individually in sterile recipients or sterile plastic pouches. Gloves were changed between each collection of pooled samples. The samples were transported to the laboratory in an icebox with freezer packs within 2 h of collection. Bacteriological analysis was usually initiated on the day of sampling but sometimes stored in the fridge at 4 °C for analysis the following day.

2.5. Bacteriological analysis of samples

The bacteriological analysis of samples was carried out according to standard norm ISO 6579: 2002 (ISO (Comité international de normalisation AW/9), 2002). Twenty five grams of each pooled feces and feed sample was weighed before adding 225 mL of buffered peptone water (BPW) (Merck, Darmstadt, Germany). Each pool of drag swabs used to sample dust and surfaces was immersed separately in 100 mL BPW per swab. Each cotton-tipped swab was placed in 9 mL BPW, and 25 mL water samples were placed in sterile bags containing 225 mL buffered peptone water. Pooled feces and feed samples were mixed in a Stomacher bag for 1 min. All the samples were incubated for 18 ± 2 h at 37 °C. Two milliliters of this pre-enrichment broth was then used to inoculate a 20 mL Mueller-Kauffmann tetrathionate-Novobiocin (MKTTn) broth (AES Chemunex, Combourg, France) and 100 µL of the pre-enrichment broth was used to inoculate 10 mL Rappaport-Vassiliadis broth (Biokar, Beauvais, France). Both media were incubated for 24 h at 37 and 42 °C respectively. Following incubation, one loopful from each selective enrichment tube was streaked over two selective media – Hektoen and Xylose Lysine Deoxycholate agar plates (AES Chemunex, Combourg, France) – prior to incubation at 37 °C for 18 to 24 h. Two typical colonies of *Salmonella* were confirmed by biochemical assays on Triple Sugar Iron (Biokar, Beauvais, France) and API20E strips (BioMerieux, Marcy l'Etoile, France). *Salmonella* isolates were then serotyped by a slide agglutination test with polyvalent O and H antiserum (Bio-Rad, Marne la coquette, France). The Kauffmann–White scheme was used to serotype the different isolates (Popoff, 2001).

2.6. Antibiotic resistance analysis

Antimicrobial susceptibility was tested by the disk diffusion method on Mueller-Hinton agar (Bio-Rad) in accordance with the Clinical and Laboratory Standards Institute (CLSI) standard (CLSI, 2006). The antimicrobial agents tested were ampicillin (10 µg), amoxicillin + clavulanic acid (20, 10 µg), cephalothin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), sulphonamides (300 µg), cotrimoxazole (1.25/23.75 µg), gentamicin (10 µg), streptomycin (10 µg), kanamycin (30 µg), tetracycline (30 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), ofloxacin (5 µg) and enrofloxacin (5 µg). Zone diameters were read using the automated Osiris scanner (Bio-Rad), interpreted as per CLSI guidelines (CLSI, 2009). *Escherichia coli* ATCC 25922 was used as quality-control organism.

2.7. Statistical analyses for factors associated with Salmonella flock contamination

Data were analyzed using Epi-Info® software (Epi Info 3.5.1, Centers for disease control and prevention (CDC), Atlanta, Freeware). Only univariate analyses were performed due to the small sample. Links between a certain factor and the presence of *Salmonella* did not therefore take into account potential confounders as multivariate regression models do. However, univariate associations suggest risk indicators, and are a preliminary but necessary step in the investigation of risk factors for any disease. Statistical analyses were limited to Fisher's Exact tests and calculation of Relative Risks (RR) with their 95% confidence intervals in order to quantify the relationship between each factor and the *Salmonella* status of laying-hen flocks. Discrepancies may exist between *p*-values and 95% confidence interval boundaries because Fisher's Exact test is a non-parametric test and 95% confidence intervals are calculated using a parametric method. The significance of associations is based on Fisher's Exact test, and not on the presence or absence of 1 in the confidence intervals. Only factors with a RR ≥ 2 are presented. All tests were two-tailed and p-values < 0.05 were considered significant.

3. Results

3.1. Salmonella prevalence

Eight of the 18 flocks tested positive for *Salmonella* spp. Table 1 gives a detailed overview of the laying-hen flocks sampled. *Salmonella* contamination was higher at the end of the laying period than at the beginning or middle. The 70- to 86-wk-old hens had the highest prevalence of *Salmonella* (7/17, 41.17%), followed by the 22- to 31-wk-old hens, which had a prevalence of 20% (3/15). *Salmonella* prevalence was lowest halfway through laying (47–60 week) with 7.14% (1/14). Furthermore, of the three layer houses tested positive at the beginning of laying, *Salmonella* had been isolated at the end of laying in the previous two flocks reared in the same layer houses. However, no statistical analysis on the association of *Salmonella* prevalence and the hens' three age classes could be performed because of the relatively small number of flocks sampled.

The number of positive samples within flocks was very low. Only 19 out of 2754 samples analyzed tested positive for *Salmonella*. Thirteen isolates (0.80%) were recovered from the 1610 feces samples and four (1.24%) from the 322 dust samples. *Salmonella* was also isolated from two (0.80%) out of 250 water samples (Table 2).

3.2. Serovars encountered and antimicrobial resistance patterns

The serotyping of 19 *Salmonella* strains revealed nine serovars from eight positive flocks, but no clearly dominant serovar emerged. One serovar was isolated in five positive flocks, and two serovars were isolated in three other flocks (Table 1). The six most prevalent serovars were *S.* Enteritidis (n = 4), *S.* Kentucky (n = 3), *S.* Hadar (n = 3), *S.* Virchow (n = 2), *S.* Heidelberg (n = 2) and *S.* Manhattan (n = 2) (Table 2). *S.* Enteritidis and *S.* Virchow were each recovered from two successive flocks reared in the same two houses (flocks no. 2

Table 1
Detailed overview of *Salmonella* isolation from samples (feces, dust swabs, surface swabs, feed, water) collected from 18 laying-hen flocks in two Algerian regions.

Flock no.	N positive/ samples analyzed at hen age			
	22–31 week	47–60 week	70–86 week	*Salmonella* serovars found in positive samples
1[a]	0/61	0/61	0/61	
2[a]	3/61[b]	0/61	1/61[c]	*S.* Enteritidis
3	0/61	0/61	2/61	*S.* Manhattan
4	0/61	0/61	0/61	
5[a]	1/61[b]	0/61	1/61[c]	*S.* Virchow
6	0/61	N.A	0/61	
7	0/61	0/61	2/61	*S.* Kentucky; *S.* Typhimurium
8	N.A	N.A	0/61	
9	0/61	0/61	3/61	*S.* Heidleberg; *S.* Kentucky
10[a]	0/61	0/61	0/61	
11	0/61	0/61	1/61	*S.* Kentucky
12	N.A	N.A	0/57	
13	N.A	N.A	0/57	
14	0/57	0/57	1/57	*S.* Dublin
15	0/61	0/61	0/61	
16	0/57	0/57	0/57	
17	0/57	0/57	0/57	
18	1/57	3/57	DEP	*S.* Hadar; *S.* Albany

N.A: No Access. Restricted house access by farmers for only one or 2 sampling visits.
DEP: the henhouse was depopulated when hens reached 66 weeks old.
[a] Four henhouses sampled at the end of lay for a first flocks and at the beginning and the middle of lay in the subsequent flocks reared in the same henhouses.
[b] Presence of *Salmonella* at the beginning of laying in the second flock housed in the same layer buildings.
[c] Samples were collected from the previous flock at the end of its laying period.

and 5, respectively, Table 1): in feces from the first flocks (n = 2) and in dust samples in subsequent flocks in the same houses (n = 4).

Thirteen isolates were resistant to at least one antimicrobial. Twelve isolates exhibited resistance to nalidixic acid. Six isolates were resistant to at least three different antibiotic classes. All three *S.* Kentucky and two isolates of *S.* Hadar were resistant to ampicillin, tetracycline and streptomycin, these five isolates being resistant to cephalotin too. The three *S.* Kentucky isolates were also resistant to fluoroquinolones (enrofloxacin, ofloxacin, and ciprofloxacin were tested, $MIC_{cipro} \geq 8$ mg/L). *S.* Typhimurium was resistant to ampicillin. *S.* Manhattan, *S.* Virchow and *S.* Dublin isolates were 100% susceptible to all tested antimicrobials (Table 2).

Table 2
Sources and antibiotic resistance patterns of isolated serovars.

Source	All positive flocks (8/18)			
	No. of samples analyzed	No. of samples tested positive	Serovars identified	Resistance pattern
Feces	1610	13 (0.80%)	*S.* Enteritidis (n = 1)	[Na]
			S. Virchow (n = 1)	susceptible
			S. Kentucky (n = 3)	3 [A-CF-S-SXT-CN-T-Na-OFX-ENR]
			S. Heidelberg (n = 2)	[NA]
			S. Hadar (n = 3)	2 [A-CF-S-T-Na]
				1 [S-T-Na]
			S. Manhattan (n = 2)	2 Susceptible
			S. Albany (n = 1)	[Na]
Dust from cage	322	4 (1.24%)	*S.* Enteritidis (n = 3)	2 [Na]
				1 Susceptible
			S. Virchow (n = 1)	1 Susceptible
Water	250	2 (0.80%)	*S.* Dublin (n = 1)	1 Susceptible
			S. Typhimurium (n = 1)	[A]

No *Salmonella*-positive sample for pools of feed and surface swabs samples.
Ciprofloxacine MIC ≥ 8 μg/mL.
A — ampicillin, S — streptomycin, T — tetracycline, CN — Gentamicin, Na — nalidixic acid, OFX — ofloxacin, ENR — enrofloxacin, CF — cephalothin, SXT — cotrimoxazole.

3.3. Risk indicators associated with the Salmonella status of flocks

Six out of fifteen risk indicators were considered as pertinent indicators since their RR was ≥2. However, none was found to be significantly related to contamination ($p<0.05$; Table 3), probably because of the small population. *Salmonella* contamination of flocks was more frequent when (i) the layer house was easily accessible (RR = 3.5, CI 95% = 0.5, 25; p = 0.1), (ii) the buildings were not disinfected before pullets were loaded (RR = 2.7, CI 95% = 0.4, 16.9, p = 0.2), (iii) ventilation was poor (RR = 2.6; CI 95% = 1.03, 6.5; p = 0.08), (iv) dead hens were stored on the farms for at least 1 week (RR = 2.6; CI 95% = 1.0, 6.5; p = 0.08), (v) we observed living or dead rodents inside the henhouse on each sampling visit (RR = 2.6; CI 95% = 0.8, 7.6; p = 0.08), and when (vi) cages were dry cleaned only before pullets were loaded (RR = 2, CI 95% = 0.7, 5.3; p = 0.2) (Table 3).

Table 3
Potential risk indicators for *Salmonella* contamination of laying hen flocks.

Variables	All flocks				
	Nb of flocks	S+[a]	RR[b]	95% CI[c]	p[d]
Farm characteristics					
Presence of fence (easy access to the henhouses)					
No	12	7 (58.3%)	3.5	0.5–25	0.1
Yes	6	1 (16.7%)	1[e]		
Poultry house characteristics					
Ventilation system					
Poor [f]	5	4 (80%)	2.6	1.0–6.5	0.08
Medium–good[g]	13	4 (30.8%)	1		
Biosecurity measures					
Disinfectant used for cleaning henhouse before pullets loaded					
No	13	7 (53.8%)	2.7	0.4–16.9	0.2
Yes	5	1 (20%)	1		
Frequency of dead bird collection					
Kept on the farm at least 1 week	5	4 (80%)	2.6	1.0–6.5	0.08
Once a day	13	4 (30.8%)	1		
Rodents observed in the layer house during visit					
Yes	7	5 (71.4%)	2.6	0.8–7.6	0.08
No	11	3 (27.3%)	1		
Dry cleaning of cages before pullets loaded					
Yes	6	4 (66.7%)	2	0.7–5.3	0.2
No	12	4 (33.3%)	1		

[a] Number of *Salmonella*-infected flocks (%).
[b] Relative risk obtained by Fisher's test.
[c] Confidence interval.
[d] Fisher's test.
[e] Protective class was always considered the reference class.
[f] Static ventilation/insufficient windows so absence of fresh air associated with high temperatures inside the layer house (>30 °C).
[g] Dynamic or natural ventilation based on a sufficient number of windows/temperatures were maintained between 20 °C–27 °C.

4. Discussion

4.1. Prevalence

To our knowledge, this is the first epidemiological study of *Salmonella* contamination in laying-hen flocks ever carried out in the Annaba and Eltarf regions of Algeria. Prior to this research, very little information was available on *Salmonella* prevalence in Algerian poultry farms. Although the study by Elgroud et al. (2009) and the present study are not strictly comparable in terms of breeding type, housing system and methodology of sampling, it might be worth pointing out that the prevalence estimated by Elgroud et al. (2009) emphasizes quite a high level of *Salmonella* contamination in Algerian poultry farms. The results of our own study show that eight flocks (44.4%) tested positive for *Salmonella* during the laying period. Within the European Union, a baseline study conducted by EFSA between October 2004 and September 2005 on laying-hen flocks at the end of the rearing period showed an average prevalence of *Salmonella*-positive flocks of 30.8% (EFSA, 2007b). The *Salmonella* prevalence in this kind of flock varied widely between member states: 17.9% in France (Huneau-Salaün et al., 2009), 31.08% in Belgium (Namata et al., 2008), 79% in Portugal (Hald, 2008) and 0% in Sweden and Luxembourg (Van Hoorebeke et al., 2010).

Our results should be interpreted with caution because of the relatively small number of sampled flocks, and because the sample was not randomized from the Algerian laying-hen population, which restricts comparability with research in developed countries on *Salmonella* prevalence in egg-laying flocks. It should nevertheless be emphasized that our study is practically exhaustive with respect to these two regions, and that poultry breeding in these regions is similar to poultry breeding nationwide. We consequently consider that our study may be representative enough for a preliminary estimation of the prevalence of *Salmonella* contamination in Algerian laying-hen flocks. It should also be noted that the same person was responsible for visiting all the farms and collecting all the data, which should make data collection and interpretation more uniform.

Our results show that *Salmonella* prevalence was higher at the end of the laying period than at the beginning or middle. It is well known that the chance of detecting *Salmonella* in a flock increases as the hens get older (Namata et al., 2008). It can be attributed to the fact that the layers are physiologically stressed when reared in close confinement in caged systems and particularly during egg production. Several authors have reported that the stress factors such as the onset of lay, high temperatures, induced molting, final stages of the production period or transportation to the slaughterhouse can cause recurrences of *Salmonella* excretion (Golden et al., 2008; Humphrey, 2006; Van de Giessen et al., 2006). Furthermore, in this work, two out of three henhouses that tested positive at the beginning of laying had been declared positive at the end of the previous flocks' laying period. Four *Salmonella* isolates were recovered from dust samples when subsequent flocks were housed in the same henhouses. It was previously thought that dust samples can give more accurate information on the layer house's past *Salmonella* status (Arnalod, Carrique-Mas, & Davies, 2009). The result of the current study clearly corroborates this finding.

The proportion of positive samples in our study is quite low—only 19, or 0.68%. Despite the relatively short transportation time and the larger number of samples taken in our study, *Salmonella* was only found in a few sampled environments. However, several studies have shown that the intensive sampling of laying-hen environments is regarded as an effective method for detecting *Salmonella* in laying-hen flocks with a low contamination level (Aho, 1992; Carrique-Mas et al., 2009; Musgrove & Jones, 2005). The low proportion of *Salmonella* in seven pooled samples in this study may be explained by the low degree of shedding at the time of sampling and/or the intermittent excretion of *Salmonella* by infected birds (Van Immerseel et al., 2004). Furthermore, this finding indicates that this sampling protocol (limited to pooled feces, dust and environmental samples) used in our study is not sufficiently sensitive in flocks where the bacterium is only shed in very small amounts, or not shed at all. We might suppose that in layer houses which are apparently *Salmonella*-negative, on the basis of the results from the bacteriological analysis of all pooled samples, a proportion of the hens may still carry the pathogen without shedding it, leading to an underestimation of the actual prevalence of *Salmonella* in the studied flocks. This is suggested by the data reported by Van Hoorebeke et al. (2009), which indicate that the post-mortem examination of hens (cecal and/or ovary/oviduct samples) is the most accurate test to determine the infection status of laying-hen flocks with a low *Salmonella* contamination level.

4.2. Serovars and antibiotic resistance

The distribution of *Salmonella* serovars identified in the present study was particularly heterogeneous. *Salmonella* Enteritidis was the most commonly isolated serovar in terms of number of isolates ($n = 4$), but it was recovered in only one henhouse. A single isolate of *Salmonella* Typhimurium was recovered during our investigation. Nationally, *S.* Enteritidis and *S.* Typhimurium serotypes are the most frequently isolated from poultry samples. In a study conducted between 1998 and 2002 by the Pasteur Institute in Algiers on 51,826 samples from all types of poultry farms including hens, eggs and turkeys, 112 out of positive strains belonged to the serovar *S.* Enteritidis (Aboun et al., 2003). In 2006, 26 confirmed outbreaks involving *Salmonella* Enteritidis and Typhimurium were reported in northern regions of the country in different poultry farms, including laying-hen flocks (Anonymous, 2006), and 25 confirmed outbreaks in 2008 involved *Salmonella* Enteritidis (Anonymous, 2009). During this study (March 2008 to November 2009), 50 strains of *S.* Typhimurium and eight strains of *S.* Enteritidis were recovered in human patients from both Annaba and Eltarf regions. Unfortunately, no information is available about the possible link between these clinical cases and egg consumption. It is also worth mentioning that many cases may not be reported because the ill person either does not visit a doctor or no sample is obtained for laboratory tests. Voetsch et al. (2004) estimated that for each laboratory-confirmed case of *Salmonella*, there are 38 cases that are missed through a lack of surveillance. In the European Union, *S.* Enteritidis was the most commonly reported serovar in 2008 (58.0% of all confirmed cases), followed by *Salmonella* Typhimurium (21.9%) (EFSA, 2010). The number of *S.* Enteritidis cases is tending to decrease in EU member states, increasing the relative proportion of *S.* Typhimurium cases.

In our research project, *Salmonella* Kentucky was recovered from three different laying-hen flocks, two isolates of *S.* Manhattan and one isolate of *S.* Albany were found in a single flock respectively. The serotypes *S.* Kentucky, *S.* Manhattan, and *S.* Albany are not usually isolated from poultry samples in Algeria. These isolates may have been acquired from the environment or animals' sources indicating the diversity of the potential reservoirs of nontyphoidal *Salmonella*. Unfortunately, no investigations have been conducted to identify the origin of contamination. A single strain of *Salmonella* Dublin was isolated once in a water sample in our study. This serotype is rarely encountered in poultry, and more frequently associated with bovine production. Its presence may be the consequence of the presence of other animals on the farm and the absence of precautionary measures by farm staff. Serovar diversity could suggest a plethora of sources: the environment, feed, water, easy access to other animal species, carried by farm workers and more.

In this study, the number of isolates resistant to at least three antimicrobials is relatively small and could indicate that the use of antibiotics in laying-hen flocks remains moderate. None of the flocks was treated with antimicrobials according to farmers' statements. The overall limited antimicrobial usage in egg-producing laying hens in our study is in accordance with previous studies on laying hens

(Kojima et al., 2009; Schwaiger, Schmied, & Bauer, 2008, 2010), which showed that the levels of antimicrobial resistance in laying hens are relatively low compared to broilers, pigs and, to a lesser extent, cattle (Bywater et al., 2004; De Jong et al., 2009; Persoons et al., 2009). In the current study, seven *Salmonella* isolates were found to be resistant to tetracycline and streptomycin. Resistance to these antimicrobials is commonly observed among *Salmonella* isolates and has been reported by several studies in poultry products (Manie, Khan, Brozel, Veith, & Gouws, 1998; Nayak et al., 2004). In the present study, resistance to nalidixic acid was detected in twelve isolates belonging to five serotypes, and resistance to ciprofloxacin was only detected in three isolates of *S.* Kentucky with MIC≥8 mg/L. The presence of isolates resistant to fluoroquinolones is more worrying, as this class of antimicrobials is often used to treat severe human salmonellosis. To our knowledge, this is the first isolation of ciprofloxacin-resistant *S.* Kentucky in Algeria, but no investigation has been conducted to identify the source of contamination. This coincides with findings of similar ciprofloxacin-resistant isolates of *S.* Kentucky isolated by the Pasteur Institute in Paris at the same time and related to travelers returning from northeast and eastern Africa (Le Hello et al., 2010). In poultry, this ciprofloxacin resistant Kentucky clone was isolated from chickens and turkeys in three noncontiguous African countries (Ethiopia, Morocco, and Togo), suggesting that poultry may be an important vehicle for infection by this strain (Le Hello et al., 2010). A possible source of contamination of African poultry could be the aquaculture farms in the North–East or East of Africa, as they use antimicrobial agents intensively. This ciprofloxacin-resistant *S.* Kentucky clone may have spread to poultry through fish meal feeds originating from these aquaculture farms. In our investigation, the antimicrobial resistance tests revealed a resistance to cephalotin in five isolates from two serotypes, *S.* Hadar and *S.* Kentucky. No isolate resistant to third-generation cephalosporin has been isolated, unlike human isolates from these regions (data not shown) that showed a considerable increase in resistance to third-generation cephalosporins in hospitals.

4.3. Risk Indicators associated with the Salmonella status of flocks

In accordance with documented research, we identified six risk indicators with RR values ≥2. None were statistically significant, but we cannot rule out a lack of statistical power due to our restricted sample.

The absence of a fence at the entrance of henhouses appears to influence the *Salmonella* status of laying-hen flocks. *Salmonella* was found more frequently on farms where non-essential visitors have easy access to the layer houses (58.3% vs 16.7%). A fence also seems to be effective in protecting the buildings from access by domestic or wild animals. A fence also restricts admittance of persons, especially visitors not on the farming staff, who could be indirect mechanical vectors of the bacteria, as seen in previous studies (Cardinale, Tall, Gueye, Cisse, & Salvat, 2004; Hald, Wedderkopp, & Madsen, 2000; Hunneau-Salaün, Denis, Balaine, & Salvat, 2007).

The absence of disinfection before new pullets were loaded appeared to be a risk indicator and revealed a potential link to *Salmonella* contamination. Seven out of 13 flocks where no disinfectant was used were found to be *Salmonella*-positive (53.8% vs 20%). This result might indicate an inadequate cleaning and disinfection procedure. The persistence of *Salmonella* contamination between two successive flocks housed in the same building was probably a consequence of this ineffective cleaning procedure. Research on broiler chickens (Baggesen, Olsen, & Bisgaard, 1992; Lahellec et al., 1986) has demonstrated that ineffective cleaning and disinfection could be the predominant risk factor of *Salmonella* contamination.

Salmonella is found more often in layer houses where dead birds are stored on the farm for at least 1 week (80% vs 30.8%). Keeping the dead birds on the farms may increase the number of insects, flies, beetles and pests which can act as a vector for several diseases and pathogenic bacteria. For the farms evaluated in this study, four out of five henhouses where numerous flies were observed inside the layer houses during sampling visits tested positive for *Salmonella*. This observation underlines the importance of biosecurity measures to protect birds from infections and their dissemination. It is also in agreement with previous studies (Craven et al., 2000; Hoover, Kenney, Amick, & Hypes, 1997; Liljebjelke et al., 2005).

The layer house ventilation system appeared to be a risk indicator for the flock's *Salmonella* contamination in our study. It may be indirectly an evidence of a potential link between high temperature inside the henhouses, due to the poor ventilation, and the presence of *Salmonella*. In the current study, 16 henhouses were ventilated naturally by windows and doors, and only two featured a mechanical ventilation system. Of the five henhouses where there were not enough windows and where air renewal was insufficient and the temperature high (>30 °C), four were contaminated (80% vs 30.8%). The lack of fresh air circulating inside the henhouse leads to high inside temperatures, especially in summer. High temperatures can cause agitation and stress in hens, which can subsequently lead to *Salmonella* excretion. Stress has already been shown to have an immunosuppressive effect in laying hens (El-Lethey, Huber-Eicher, & Jungi, 2003; Humphrey, 2006), with possible negative consequences on *Salmonella* shedding.

The observed presence of living or dead rodents inside the henhouses appears to influence the *Salmonella* contamination of layer flocks in our study (71.4% vs 27.3%). It has been reported that conventional battery cage housing is a more attractive environment for pests because the caged hens cannot move around as much (Carrique-Mas, Marin, Breslin, McLaren, & Davies, 2009). In our study, rodents inside the layer house may be a risk factor because they can act as vectors for *Salmonella*. The number of flocks studied is too small to confirm this tendency. However, studies carried out on broiler chickens and laying-hen flocks identified rodents as a potential source of *Salmonella* contamination and persistence in poultry houses (Henzler, Ebel, Sanders, Kradel, & Mason, 1994; Henzler & Opitz, 1992).

The dry cleaning of cages appears to influence the *Salmonella* contamination of flocks in our study (53.8% vs 20%), and reveals a potential link to *Salmonella* prevalence in layer houses. It is a well-known fact that the cage systems are not dismantled and are consequently extremely difficult to clean and disinfect because of the restricted access to cage interiors, feeders, egg belts and so forth (Carrique-Mas, Breslin, et al., 2009; Davies & Breslin, 2003). In the current study, the fact that two *Salmonella* serovars were present in dust samples from cages in the second hen flocks reared in the same layer houses was probably the result of this difficulty in efficiently cleaning the cages.

5. Conclusion

The current study provides baseline data on the *Salmonella* contamination of Algerian laying-hen flocks and some useful information on the presence of *Salmonella*, antimicrobial resistance and the associated risk factors for laying-hen farms. We showed that eight flocks tested positive for *Salmonella* among 18 flocks sampled and that layer life stages also affected the prevalence of *Salmonella* recovered from the seven pooled samples. The results of this study also illustrate that, despite using an extended sampling protocol on flock (3 pooled feces samples, 2 pools of dust and environmental swabs, 2 pools of water and feed samples), the proportion of positive samples remains very low (19/2754). We also revealed the presence of multiple *Salmonella* serovars, and a small proportion of antibiotic-resistant isolates, which may suggest a moderate use of antibiotics in Algerian laying-hen flocks. Unfortunately, no published data is available regarding the use of antimicrobial on Algerian farms. In

the present study, we report here the presence of ciprofloxacin-resistant *S*. Kentucky in the studied flocks that coincide with its emergence in other countries throughout Africa and the Middle East. However, the low number of isolates that may be due to limitation of the Algerian area studied does not allow assessing the potential introduction of ciprofloxacin Kentucky clone at a national level. Six risk indicators were identified as being potentially linked to the *Salmonella* contamination of laying-hen houses. Most have already been reported as potential risk factors for *Salmonella* contamination in laying-hen flocks. In the future, these initial findings may be used as the basis for national epidemiological studies to determine the prevalence of *Salmonella* spp. in Algerian laying-hen flocks and to investigate the risk indicators implicated in *Salmonella* spp contamination, the different serovars present and even any relationship between egg production and human outbreaks of *Salmonella*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.foodres.2011.05.027.

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