Assessment of *Salmonella* spp. in feces, cloacal swabs, and eggs (eggshell and content separately) from a laying hen farm

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ABSTRACT Microbial pathogens of the genus *Salmonella* are among the leading causes of foodborne illness in the world. The present study was done on a laying hen farm with a *Salmonella enterica* serovar Enteritidis-positive result according to the testing specified by European regulation 2160/2003. The aim of this study was to compare the *Salmonella* contamination on a laying hen farm with the *Salmonella* presence in the hen eggs. The strains were isolated by ISO method 6579:2002 (standard method for the detection of *Salmonella* spp. in the European regulation for food and animal feeding stuffs, animal feces, and environmental samples from the primary production stage, including poultry farms) and were confirmed as *Salmonella* Enteritidis by the Kauffmann-White method. In addition, strains were compared with genomic macrorestriction followed by pulsed-field gel electrophoresis. Four types of samples, namely, feces (n = 50), cloacal swabs (n = 150), eggshells (n = 50), and egg contents (n = 50), were taken from each of 50 randomly selected battery cages. Results demonstrated that feces (92%) were the most positive sample, followed by eggshells (34%) and cloacal swabs (4%). No *Salmonella* spp. were detected in the egg contents. Our results show that a *Salmonella* Enteritidis-positive result on a laying hen farm, according to the testing specified by European regulation 2160/2003, did not imply the presence of the pathogen in the egg contents. Additionally, *Xba*I-digested genomic DNA of *Salmonella* Enteritidis strains isolated from several samples resulted in the same pattern, so were probably of the same origin.

Key words: cloacal swab, egg, feces, hen, *Salmonella*, serotype

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INTRODUCTION

Salmonella has long been recognized as an important zoonotic pathogen of economic significance in animals and humans. The genus *Salmonella* is currently divided into 3 species: *Salmonella enterica*, *Salmonella bongori*, and *Salmonella subterranea*. *Salmonella enterica* is further divided into 6 subspecies, and most *Salmonella* belong to the subspecies *S. enterica* ssp. *enterica*. More than 2,500 serovars of zoonotic *Salmonella* exist, and the prevalence of the different serovars changes over time (EFSA, 2009). Overall in the European Union, *S. enterica* serovar Enteritidis (*Salmonella* Enteritidis) and *S. enterica* serovar Typhimurium (*Salmonella* Typhimurium) are the serovars most frequently associated with human illness. Human *Salmonella* Enteritidis cases are most commonly associated with the consumption of contaminated eggs and broiler meat, whereas *Salmonella* Typhimurium cases are most often associated with the consumption of contaminated pig, poultry, and bovine meat. *Salmonella* Enteritidis and *Salmonella* Typhimurium accounted for 64.5 and 16.5% of all reported cases of human salmonellosis in Europe (EFSA, 2009).

The purpose of European regulation 2160/2003 (European Commission, 2003) is to ensure that proper and effective measures are taken to detect and control *Salmonella* and other zoonotic agents at all relevant stages of production, processing, and distribution, particularly at the level of primary production (including in the feed) to reduce their prevalence and the risk they pose to public health.

Currently, tests are carried out in accordance with the International Standard ISO 6579:2002 (International Organization for Standardization, 2002a) for food and animal feeding stuffs or by using the ISO 6579:2002 annex D method (International Organiza-

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tion for Standardization, 2002b) for animal feces and environmental samples from the primary production stage. Once strains are isolated, they can be serotyped according the Kauffmann-White method (Grimont and Weill, 2007). Additionally, to study the origin of the isolated strains, the genetic characterization of colonies can be performed to compare the isolated strains from the same serovar.

The aim of this study was to do a qualitative comparison of the *Salmonella* contamination on a laying hen farm (*Salmonella* Enteritidis-positive result according to European regulation 2160/2003; European Commission, 2003) with the presence of *Salmonella* in their eggs.

MATERIALS AND METHODS

Laying Hen Farm in the Study

A laying hen farm with a *Salmonella*-positive result according to European regulation 2160/2003 (European Commission, 2003) was selected. The farm had 150,000 twenty-seven-week-old Lohmann Brown commercial laying hens in 4 houses. The studied house contained 65,000 hens distributed into 12 hens per battery cage, stacked 6 cages high. Both official veterinary and farmer feces sampling at 24 wk of age led to a positive *Salmonella* Enteritidis result.

During the sampling of 27-wk-old hens, the temperature was 22°C, with 16 h of daily lighting. The cage surface for each bird, centimeters of feeding space, and floor inclination were in accordance with Spanish animal care guidelines (Annex II of RD 3/2002; Ministerio de Medio Ambiente y Medio Rural y Marino, 2002). Birds are vaccinated 2 times against salmonellosis with live vaccines in the drinking water (*Salmonella* Enteritidis; wk 2 and 8) on a conventional rearing farm.

Sampling

Before slaughter at the hen age of 27 wk, 4 types of samples, namely, feces (n = 50), cloacal swabs (n = 150), eggshells (n = 50), and egg contents (n = 50), were simultaneously taken from each of 50 randomly selected battery cages.

First, a collective sample of 150 g or more of feces (simulating farmer or official controls) was taken from the feces belt using a sterile tongue depressor and a different pair of gloves for each cage to avoid cross-contamination. Each sample was transported to laboratory in a 500-mL sterile jar. Second, each cloacal swab was analyzed independently. A sterile swab was inserted into the cloaca of the hen and then turned slowly to take the fluid sample. Until delivery to the laboratory, the swab was kept in a sterile tube containing 10 mL of buffered peptone water. Finally, for each cage, 6 hen eggs 1 d from the date of laying were collected from the egg belt. The egg contents and the outer surface of the egg were analyzed independently. A different pair of gloves was used for sampling each cage. All the samples were transported to the laboratory approximately 2 h after collection, stored under refrigeration, and processed on the day of sampling.

Microbiological Analysis

The isolation and identification method for the samples consisted of nonselective preenrichment followed by selective enrichment, isolation, and biochemical identification, all steps according to ISO method 6579:2002 (International Organization for Standardization, 2002a). Cloacal swabs, feces, and samples of eggshells were analyzed by a modification of this ISO method (Annex D; International Organization for Standardization, 2002b), using semisolid modified Rappaport-Vassiliadis medium (**MSRV**; Difco, Madrid, Spain) as the selective enrichment medium.

First, preenrichment of the samples in nonselective medium was done at a dilution of 1:10 in buffered peptone water. This dilution was incubated at 37 ± 1°C for 18 ± 2 h for all the samples. This dilution was performed differently according to the sample tested.

For feces, the sample was homogenized and 25 g of the mixture was weighed and added to 225 mL of buffered peptone water, after which the sample was homogenized again. For cloacal swabs, the sample in 10 mL of buffered peptone water was considered a dilution of 1:10. For eggshells, the surface of the 6 eggs was considered a sample. Each group of 6 eggs was kept in contact with 90 mL of buffered peptone water for 10 min. From these samples, 100 μL of the preenriched culture was inoculated on MSRV. The MSRV was incubated at 41.5 ± 1°C for 24 ± 3 h. If a plate was negative after 24 h, it was incubated for a further 24 ± 3 h. Positive plates showed a gray-white and turbid zone extending out from the inoculated drop. The turbid zone was characterized by a white halo with a clearly defined edge. Two solid selective media, xylose lysine deoxycholate agar (Oxoid, Madrid, Spain) and xylose lysine tergitol-4 agar (Oxoid), were inoculated and incubated at 37 ± 1°C, and then examined after 24 ± 3 h.

For egg contents, the surfaces of 6 eggs were flamed with ethanol (each egg was dipped in 96% ethanol and then flamed) to avoid cross-contamination. The flamed eggs were cracked and the contents were collected in a sterile bag for homogenization. A 225-mL quantity of buffered peptone water was added to 25 g of the homogenized egg contents, and then the contents were homogenized again. In the next step, samples of the egg contents were processed differently from the other samples. Thus, 100 μL of the preenriched culture from the egg contents was transferred to a tube containing 10 mL of Rappaport-Vassiliadis medium with soy (**RVS**; Scharlau, Sentmenat, Spain), and 1 mL was transferred to a tube containing 10 mL of Müller-Kauffmann tetrathionate/novobiocin (**MKTTn**) broth (bioMérieux, Madrid, Spain). The RVS broth was incubated at 41.5 ± 1°C and the MKTTn broth was incubated at 37 ±

1°C for 24 ± 3 h. From the cultures of the RVS and MKTTn broths, 2 selective solid media were inoculated. These solid media were xylose lysine deoxycholate agar and xylose lysine tergitol-4 agar, which were incubated at 37 ± 1°C for 24 ± 3 h.

Later, 5 colonies of presumptive *Salmonella* isolated were streaked from the selective plating media onto nutrient agar (Scharlau) and then incubated at 37 ± 1°C for 24 ± 3 h. Finally, biochemical confirmation of *Salmonella* spp. was performed with an API 20E identification kit (bioMérieux).

According to ISO method 6579:2002 (International Organization for Standardization, 2002a), after biochemical confirmation of the colonies suspicious for genus *Salmonella* spp., serotyping has to be done for somatic (O), flagellar (H), and capsular (Vi) antigens by the plate agglutination test according to the Kauffmann-White scheme.

The characterization of the strains consisted of serotype determination and genetic material analysis. Strains confirmed as *Salmonella* Enteritidis by the plate agglutination test were compared using genomic macrorestriction followed by pulsed-field gel electrophoresis (**PFGE**). The selected strains were from feces (cages 4, 15, and 24), swabs (cages 12 and 48), and eggshells (cages 16, 41, and 44).

Each studied strain was cultured in Luria-Bertani medium and then incubated in an aerobic atmosphere at 37°C for 18 h. Deoxyribonucleic acid for the PFGE experiments was extracted as described by Ribot et al. (2006). Total DNA digestion was performed with the enzyme *Xba*I (Roche Diagnostics, Sant Cugat del Vallès, Spain). Pulsed-field gel electrophoresis was performed on a Chef-DR III apparatus (Bio-Rad Laboratories, El Prat de Llobregat, Spain). An analysis of the similarity of the obtained profiles was done using the software package Fingerprinting II (Bio-Rad Laboratories). A strain of *Salmonella* Enteritidis (LK5) from the Universidad Autónoma de Barcelona was used as a stability and reproducibility control of the technique. Bands obtained from the digestion of the strains with the restriction enzyme *Xba*I (Roche Diagnostics) and PFGE were compared. Electrophoresis conditions, gel staining, and digital image acquisition were performed as described previously by Shahada et al. (2007). Analysis and interpretation of the PFGE TIFF images was carried out as explained previously (Shahada et al., 2007).

Statistical Analysis

Data on the detection of *Salmonella* spp. in each sample (feces, swabs, egg contents, and eggshells) were subjected to a chi-squared test with Statgraphics Plus 5.1 software (Manugistics Inc., Dallas, TX)

RESULTS AND DISCUSSION

Table 1 shows *Salmonella* spp. detection in the 4 different types of samples studied. Significant differences ($P < 0.05$) were found in positivity to *Salmonella* spp. among the types of samples. Feces (92%) were the most positive, followed by eggshells (34%) and cloacal swabs (4%), whereas no *Salmonella* spp. were found in the egg contents. Digestion of the genomic DNA of the isolated *Salmonella* Enteritidis using the enzyme *Xba*I followed by PFGE permitted the demonstration of identical profiles (Figure 1). These results confirmed that the *Xba*I-digested genomic DNA of *Salmonella* Enteritidis strains isolated from several samples on a laying hen farm resulted in a unique pattern. According to Ribot et al. (2006), PFGE has established itself as the "gold standard" for subtyping foodborne bacterial pathogens.

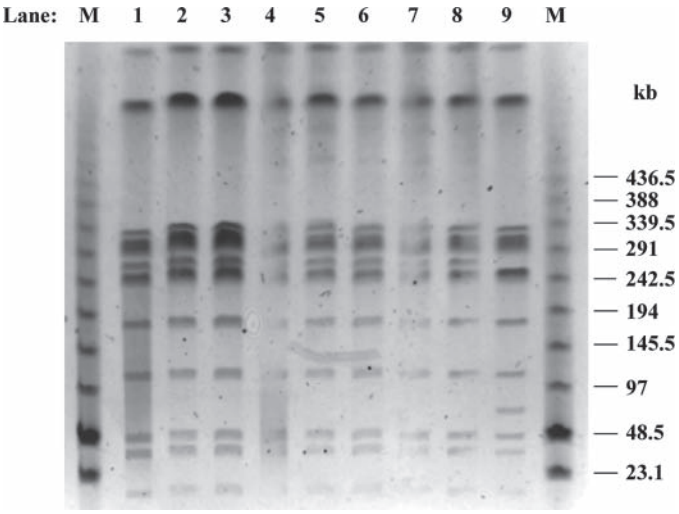

Figure 1. Pulsed-field gel electrophoresis (PFGE) patterns for *Xba*I-digested genomic DNA of *Salmonella enterica* serovar Enteritidis strains obtained from several samples on a laying hen farm. Lanes 1 and 2, *Salmonella* Enteritidis from cloacal swabs; lanes 3 to 5, *Salmonella* Enteritidis from eggshells; lanes 6 to 8, *Salmonella* Enteritidis from feces; lane 9, *Salmonella* Enteritidis (LK5); M, lambda ladder marker for PFGE.

Flock size, housing, and sampling are some factors to consider in *Salmonella* contamination. Concerning flock size, a larger flock size increases the number of susceptible birds (Mollenhorst et al., 2005). Moreover, large-sized poultry houses are more often located on farms where several poultry houses are linked to egg-packing plants by means of a common egg conveyor (Namata et al., 2009).

Concerning the housing of hens and the sampling procedure, Garber et al. (2003) found that rearing pullets on the floor instead of in cages increased the risk of

Table 1. *Salmonella* spp. detection in different types of samples (mean in % positivity of *Salmonella* spp.)

Sample	n	Positivity (%)	SE
Feces	50	92.0[a]	3.9
Cloacal swabs	150	4.0[b]	2.8
Eggshells	50	34.0[b]	6.8
Egg contents	50	0.0[c]	0.0

[a–c]Means in the same column with different superscripts are significantly different by chi-squared test ($P < 0.05$).

infection, whereas Namata et al. (2008) reported that the on-floor housing of laying hens appeared to have a protective effect. According to Valancony et al. (2001), cage poultry houses are difficult to clean and disinfect. *Salmonella* contamination has been shown to be more persistent in successive flocks housed in cages than on the floor because of poor cleaning standards and disinfection on cage farms (Davies and Breslin, 2003) or in the surroundings of the premises (Davies and Wray, 1996). Huneau-Salaün, et al. (2009) reflected that pooling feces seemed to be a less sensitive sampling method for *Salmonella* detection than dust samples or boot swabs, as described previously by Skov et al. (1999b) and Buhr et al. (2007) in on-floor broiler flocks. The relative resistance of *Salmonella* to desiccation might explain the higher probability of isolating *Salmonella* from dust samples than from pooled feces samples. In feces, the competitive flora seem to play an important role (Davies and Wray, 1996).

The higher risk of contamination in caged flocks is probably due to a failure to properly clean and disinfect the poultry house. The higher risk of contamination in caged flocks has been reported in several European countries, including Belgium (Namata et al., 2008), Denmark (Skov et al., 1999a), France (Chemaly et al., 2009), Germany (Methner, 2005), and others (EFSA, 2009).

In the cloaca of hens, the presence of *Salmonella* was lower than in the feces and eggshells. The lower recovery of *Salmonella* from cloacal swabs was probably due to the main *Salmonella* isolated from feces and eggshells coming from improperly cleaned houses and an improperly disinfected environment. El-Tras et al. (2010) reported that the cloaca are an important location involved in the later infection of the egg. Thomas et al. (2009), who inoculated hens with *Salmonella* Enteritidis, estimated the average generation time between colonization of "primary" hens and colonization of contact-exposed hens to be 7 d. A flock of 20,000 hens would reach a maximum colonization level of 92% within 80 d after colonization of the first hen. However, Van Hoorebeke et al. (2009) analyzed cloacal swabs and cecal contents and observed that fecal sampling underestimated the actual prevalence of *Salmonella* in laying hen flocks based on the results of the official monitoring program. Okamura et al. (2001) suggested that *Salmonella* Enteritidis has a specific advantage over the other *Salmonella* serovars because of its capacity to colonize the vaginal tissues of hens, and this higher affinity of *Salmonella* Enteritidis to the vagina may play a significant role in the production of many *Salmonella* Enteritidis-contaminated eggs.

In eggshells, *Salmonella* was present in 34% of the studied samples. El-Tras et al. (2010) observed that the risk for eggshell contamination was highly probable in laying hen flocks infected with *Salmonella* Enteritidis, at >30%. According to De Reu et al. (2006), the bacterial penetration of the eggshell is due to several factors, including specific gravity and conductance. Jones and Curtis (2002), using whole eggs and *Salmonella* Enteritidis, suggested that bacterial contamination of air cells, shell membranes, and egg contents was more easily achieved in eggs from older hens than from younger hens.

In egg contents, we did not detect the studied microorganism. The low and sporadic incidence of egg contamination was probably because of the protective effect of the egg's complex system of membrane barriers and the antibacterial components of the albumen. Penetration of the shell by *Salmonella* Enteritidis has been suggested (Nascimento et al., 1992; Schoeni et al., 1995), and the *Salmonella* Enteritidis, *Salmonella* Typhimurium, or *Salmonella* Heidelberg present in feces could penetrate to the interior of eggs and grow during storage.

In conclusion, the qualitative detection of the pathogen at high levels of contamination (92%) in laying hen feces did not imply its presence in the egg contents. Results of this study could help us to evaluate how *Salmonella* infection at the primary production stage contributes to *Salmonella* infection in humans, to combat wrong domestic handling of eggs. According to these results, it would be more useful to evaluate (by counting the number of bacteria) the presence of *Salmonella* spp. in laying hen feces quantitatively instead of qualitatively to establish a real correlation with the presence of the pathogen in egg contents.

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