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RESEARCH ARTICLE

Seroprevalence and Microbiological Monitoring in Eggs for *Salmonella enterica* Serovar *Enteritidis* and *Salmonella enterica* Serovar *Typhimurium* in Ornamental Chicken Flocks in Italy

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ABSTRACT

Few data are available about the prevalence of *Salmonella enterica* serovar *Enteritidis* (*S.E.*) and *Salmonella enterica* serovar *Typhimurium* (*S.T.)* in ornamental poultry in Italy. The aim of this study was to investigate the seroprevalence for *S.E.* and *S.T.* using serological tests and the prevalence of *Salmonella* spp. in eggs by culture methods. For this purpose, 240 serum samples and 216 eggs were sampled from asymptomatic and unvaccinated ornamental hens reared in 24 farms, located in 8 different Italian regions. As screening test, a *Tube Serum Agglutination* test (*TSA*) was performed on 231 out of 240 serum samples. Four out of 24 farms (16.67%) were serologically positive for *Salmonella* spp. for a total of 10 samples. These positive samples were confirmed using an *ELISA* test and the results show that 5/231 (2.16%) and 7/231 (3.03%) serum samples were positive for *S.E.* and *S.T.* respectively, and 2/231 (0.87%) for both serotypes. Among all farms, 2/24 (8.33%) were positive for *S.E.* and 4/24 (16.67%) for *S.T.* The analysis of eggs using culture methods gave negative results for both yolk and shell pools (0/48, 0.0%). The seroconversion associated with exposure to *S.E./S.T.* in ornamental poultry, poses a potential public health problem. This study confirms that *S.E.* and *S.T.* are widespread in studied backyard poultry farms as asymptomatic form, and animals as potential reservoirs of *Salmonella*. It is necessary to inform farmers that a regular and periodic control of animals, eggs or meat, is very important to prevention of *Salmonella* foodborne infections and their spread.

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INTRODUCTION

Salmonellosis is a significant zoonotic disease with a considerable economic impact on the production of eggs and in general, on the whole poultry industry. According to the European Food Safety Agency (EFSA), *Salmonella* spp. is one of the top pathogen agents involved and more distributed in European Union (EU) in foodborne outbreaks (EFSA, 2008; WHO, 2017), and is the third cause of death among food transmitted disease (Ferrari *et al*., 2019). Some serovars, such as *Salmonella Pullorum* and *Salmonella Gallinarum* are typical of birds, causing disease in animals but rarely in humans, while others such as *Salmonella enterica* serovar *Enteritidis* (*S.E.*) and *Salmonella enterica* serovar *Typhimurium* (*S.T.*) are able to infect a broad range of hosts (Ferrari *et al*., 2019) *S.E.* and *S.T.* are the serovars more associated with gastrointestinal illness in humans, but also other serovars can cause disease and represent a public health issues, such as *S. Infantis* (Vieira *et al*., 2008; EFSA, 2010). Across the EU, 32% of *Salmonella* cases reported are caused by *S.E.*, 16% from *S.T.* and 6% caused by other *Salmonella* species, especially from *S. Infantis*. In Italy, according to the reports of EFSA (2018, 2019), the percentage of positivity for zoonotic *Salmonella* spp. such as *S.T.* and *S.T.* monophasic variant (*S.T.* mv) in industrial

chicken flocks is 0.2%, while in laying hens is 0.3%. Overall, the prevalence of *Salmonella* spp. in poultry industry remains lower than 1%. Surveillance programs and intervention strategies to control foodborne salmonellosis have been implemented in EU Member States, although a clear evaluation of the effect of such interventions is difficult. From a public health perspective, there is inherent risk correlation between zoonotic transmission of pathogens and poultry husbandry and production. In the last years in Italy, the breeding of ornamental poultry for self-consumption of eggs and meat, for beauty competitions and to preserve local breeds, has regained popularity. Often, backyard poultry farmers have limited knowledge of bio-security practices and are not included in vaccination schedules or monitoring plans, as indicated on the Regulation 2160/2003 (European Commission, 2003) and 13.11.2013 (Ministry of Health, 2013). In particular, farms with less than 50 birds or with a number of birds between 50 and 250 (reared without commercial purposes but only for self-consumption) are not included in the National Control Plan for *Salmonella* (NCPS).

In general, the Italian legislation on Food Safety exclude all products intended for self-consumption from official controls, as indicated in the Regulation 178/2002 (European Commission, 2002). However, according to Regulation 1308/2013 (European Commission, 2013), eggs produced by rural farms can be sold in local markets or within 10 kilometres from the sites of production without weight classification, otherwise indicated in the Regulation 1234/2007 (European Commission, 2007) and 589/2008 (European Commission, 2008). To our knowledge, there are limited information about the prevalence of *Salmonella* spp. in ornamental poultry flocks in Italy, also by the scarcity of data that would be obtained from an adequate monitoring plan. In other countries, such as in South Australia in a study conducted by Manning *et al*. (2015), 30 backyard poultry flocks were screened for *Salmonella* spp. and 4 tested flocks resulted positive. The overall *Salmonella* spp. isolation rate in the study was 10.4%, with a prevalence at individual bird level of 0.02%. In Finland, *S. enterica* was only found sporadically in fecal and environmental samples of backyard poultry (Pohjola *et al*., 2016). In US, in different backyard poultry farms, 27 cases of paratyphoid *Salmonella enterica*, with 12 of the paratyphoid *Salmonella enterica* infections were attributed as the cause of mortality and an additional 15 cases were detected on general *Salmonella* surveillance and were not associated with clinical signs (Cadmus *et al.*, 2019). However, in Chile, some researchers highlighted the importance of breeding backyard poultry on the spreading of *Salmonella* serovars potentially hazardous to public health. In a study conducted by Alegria-Moran *et al*. (2017), different serovars were detected in backyard flocks which are linked to human and animal clinical outbreaks. Based on the results of the study of Trung *et al*. (2017), the majority of human non-typhoidal *S. enterica* outbreaks is the result of foodborne infections or of person-to-person transmission and *S. enterica* infections may also be acquired by environmental and occupational exposure to infected animals. In 2018, in our laboratory, after a suspected outbreak of foodborne infection, *S.E.* was isolated in homemade sweets containing mascarpone cream, a typical Italian dessert made with raw eggs produced using backyard hen's eggs (unpublished data). The aim of this study was to evaluate the seroprevalence for *S.E.* and *S.T.* in ornamental backyard hens raised in different Italian regions, associated with culture methods to detect *Salmonella* spp. in eggs produced by the tested flocks.

MATERIALS AND METHODS

Tested flocks: A total of 24 ornamental chicken farms located in 8 different Italian regions were included in this study. The poultry flocks selected were composed by less than 250 ornamentals pure breeds chickens, reared with free-range method, for beauty competitions or for meat and eggs self-consumption. These breeds maintain their reproductive activity from 3 to 7 years of age, were not subject to light or temperature conditioning and followed their biological reproductive cycle. Anyhow, the subjects that do not respect the breed standard are not suitable for beauty exhibitions and are intended to self-consumption, while eggs are incubated or used for home consumption or sold. Other avian ornamental species, such as waterfowl (goose and ducks), turkeys, guinea fowl, pigeons and peacocks were present in some farms, both multispecies and single species farms were considered. The main characteristics are summarised in Table 1.

Poultry feed: The feed was different for each farm, dry diet with cereals such as corn, oats, barley, wheat, sorghum, flour soya extract mix, or semi-solid, represented by mash, composed of cereal flours or by-products traditionally mixed with whey or warm water, vitamins and sunflower or soy oil. The animals were also fed with commercial feed in the first phases of growth from 0-80/90 days of age.

Vaccinations program and antibiotic treatments: The vaccination program for the main diseases of poultry of each tested flock is summarised in Table 1, no vaccination against *S.E.* and/or *S.T.* was performed and no antibiotics were administered in the 6 months preceding the sampling.

Sampling

Sample size: The total amount of hens in 24 farms was 1204 accounted for 75.39% on total of 1597 chickens. The hens included in the age-range between 5 month and 5 years, were 971 (80.64%). Based on the possibility of identifying a *Salmonella* infection with a prevalence of 5% and 95% of confidence level, a minimum of 58 subjects were sampled to identify at least one positive subject. The sampling size was increased to 240 blood samples.

Blood-serum samples: The study was conducted according to the veterinary clinical practices for no-experimental purposes, as mentioned in Article 2, paragraph 1, Letter b, of Legislative Decree No. 26/2014. With the voluntary consent of farmers, an aliquot of serum was used to verify the presence of *S.E.* and *S.T.* antibodies. Between December 2018 and February 2019, 10 blood samples (1.5 ml/sample) from 10 hens in reproductive state and asymptomatic, were obtained from each farm for a minimum percentage of 10% (Table 2). The serum obtained was frozen at -20°C until analysis.

Table 1: Location, poultry species, breeds reared and pathogens against which vaccination was applied in each tested farm

Farm	Regions	Poultry species and breeds	Vaccination
A	Emilia-Romagna	Chickens (Robusta Lionata, Brahma, Orpington), Ducks, Goose, Peacock	[a]ND, [b]IB, [c]FP, [d]AE, [e]IC
A1	Emilia-Romagna	Chickens (Polish, Silkie, Paduan, Brahma), Pigeons, Roul Roul	[a]ND, [b]IB, [c]FP, [e]IC, [g]MD, [i]MG
A2	Emilia-Romagna	Chickens (Faverolles, Cocin, Wyandotte) Ducks, Goose	[a]ND
B	Tuscany	Chickens (Leghorn)	[a]ND, [b]IB, [c]FP, [e]IC, [g]MD, [i]MG
B1	Tuscany	Chickens (Leghorn)	[a]ND, [b]IB
C	Lombardy	Chickens (Sultan, Wyandotte, Orpington, Dwarf chickens, Leghorn, Sussex)	[a]ND, [b]IB, [c]FP, [g]MD
C1	Lombardy	Chickens (Leghorn, Italiener, Amburgo), Ducks, Goose, Turkeys	[a]ND
C2	Lombardy	Chickens (Paduan, Polish)	[a]ND
C3	Lombardy	Chickens (Barnevelder)	[a]ND, [b]IB, [g]MD, [h]ILT
D	Piedmont	Chickens (Sicilian, Silkie, Cocin). Pheasant	[a]ND
D1	Piedmont	Chickens (Leghorn, Robusta Maculata), Pigeons	[a]ND
E	Lazio	Chickens (Brahma, Cocin, Amrock, Marans), Peacock	[a]ND, [b]IB, [c]FP, [e]IC, [g]MD, [i]MG
E1	Lazio	Chickens (Cemani, Lakenfelder, Orpington, Faverolles, Polish, Cocin, Vorwerk), Peacock, Ducks, Goose, Guinea fowl	[a]ND
E2	Lazio	Chickens (Silkie, Chabo, Serama, Leghorn)	[a]ND
E4	Lazio	Chickens (Paduan, Houdan, Pavlov, Serama), Pigeons, Ducks, Goose, Peacock, Guinea fowl	[a]ND
E5	Lazio	Chickens (Paduan, Polish, Cornish), Turkeys, Pheasant	[a]ND
E6	Lazio	Chickens (Brahma, Cemani, Amburgo, Yokohama, Cornish, Marans)	[a]ND
E7	Lazio	Chickens (Silkie, Australorp, Wyandotte, Araucana)	[a]ND
G	Trentino-Alto Adige	Chickens (Serama, Transilvania Naked Neck)	[a]ND, [b]IB, [c]FP, [e]IC, [f]IBD, [g]MD, [i]MG
H	Veneto	Chickens (Silkie)	[a]ND, [g]MD
I	Veneto	Chickens (Silkie)	[a]ND, [g]MD, [h]ILT
I1	Veneto	Chickens (Silkie, Paduan, Polish)	[a]ND, [b]IB
I2	Veneto	Chickens (Polverara)	[a]ND, [c]FP
L	Campania	Chickens (Ko-Shamo, Leghorn, Cornish, Wyandotte)	[a]ND

[a] ND (Newcastle Disease); [b] IB (Infection Brochitis); [c] FP (Fowlpox); [d] AE (Avian Encephalomyelitis); [e] IC (Infectious Coryza); [f] IBD (Infection Bursal disease); [g] MD (Marek's disease); [h] ILT (Infectious Laringotracheitis); [i] MG (*Mycoplasma gallisepticum*).

Table 2: Sampling

Farm	Region	N° hens present in a farm/N° total of chickens	N° hens in reproductive status/N° total of hens	N° blood samples (%)	N° eggs sampled
A	Emilia Romagna	50/60	39/50	10 (25.64%)	10
A1	Emilia Romagna	100/120	68/100	10 (14.71%)	10
A2	Emilia Romagna	27/38	27/27	10 (37.04%)	10
B	Tuscany	70/100	53/70	10 (18.97%)	10
B1	Tuscany	37/40	30/37	10 (33.33%)	10
C	Lombardy	93/130	63/93	10 (15.87%)	10
C1	Lombardy	46/51	46/46	10 (21.73%)	10
C2	Lombardy	60/75	60/60	10 (16.67%)	10
C3	Lombardy	29/40	29/29	10 (34.48%)	6
D	Piedmont	48/75	48/48	10 (20.83%)	10
D1	Piedmont	40/52	28/40	10 (35.71%)	10
E	Lazio	60/82	60/60	10 (16.67%)	10
E1	Lazio	80/120	60/80	10 (16.67%)	10
E2	Lazio	40/53	36/40	10 (27.70%)	6
E4	Lazio	60/80	60/60	10 (16.67%)	6
E5	Lazio	79/104	36/79	10 (27.78%)	10
E6	Lazio	53/67	39/53	10 (25.64%)	10
E7	Lazio	23/30	14/23	10 (71.43%)	10
G	Trentino Alto-Adige	23/25	20/23	10 (50.00%)	6
H	Veneto	18/33	18/18	10 (55.56%)	6
I	Veneto	19/25	19/19	10 (52.63%)	6
I1	Veneto	97/120	76/97	10 (13.16%)	10
I2	Veneto	30/37	22/30	10 (45.45%)	10
L	Campania	22/40	20/22	10 (50.00%)	10

Table 3: Serological results

Farm	[a]*TSA-S.E.* (Number Positive Samples/Total Samples)	*TSA-S.T.* (Number Positive Samples/Total Samples)	*TSA-S.E.* Inconclusive	*TSA-S.T.* Inconclusive	*ELISA-S.E.*	*ELISA-S.T.*	[b]Prev. *S.E.* (%)	Prev. *S.T.* (%)
A	0/10	0/10	0/10	1/10	0/10	1/10	0%	10.0%
B	1/10	3/10	4/10	2/10	4/10	2/10	40.0%	20.0%
E1	0/8	0/8	3/8	3/8	1/8	2/8	12.5%	25.0%
E5	0/8	0/8	0/8	2/8	0/8	2/8	0.0%	25.0%

[a]*TSA*: Tube Serum Agglutination test (Number Positive/Total Samples); [b]Prev: Prevalence.

Eggs samples: From 6 to 10 eggs were taken for each group tested, kindly provided by the farmers who supported the study and stored at room temperature. Microbiological analysis was performed in pool for each farm, analysing yolk and shell pools separately. In total, 216 eggs (Table 2) divided into 24 pools of egg yolk and shell were analysed.

Serological methods: A *Tube Serum Agglutination* test (*TSA*) was performed according to Davies (2008) in O.I.E. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Chap. 2.9.9., par. B.2.c; 2018, Chap. 2.3.11, par. B.2.1.). Positive samples were tested using the following commercial kits *ELISA*: ELISA Kit IDEXX SE Ab X2™ (IDEXX Laboratories-Westbrook, Maine, US) for *S.E.*

and ELISA Kit X-Ovoflockscreen™ (x-OvO Limited, Canergie Campus, Dunfermline, UK) for *S.T.*

Microbiological methods: The culture methods on eggs, were performed according to UNI-EN-ISO. 6579-1:2017. According to the UNI-EN-ISO.6579-1:2017 procedure, the isolation of *Salmonella* (including *S. typhi* and *S. paratyphi*) from different matrices such as eggs (shell and yolk), feed, faeces e coacal swabs, is performed in two principal steps. In the first step, for each farm, yolk and shell pools were obtained from the eggs, and adequately homogenized in stomacher. Two hundred twenty-five ml of Buffered Peptone Water-BPW (Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna, Brescia, Italy) at room temperature were added to 25 g of matrix (yolk and shell separately) and incubated at 36 °C for 18±2 hours. In the second step, two selective- enrichment liquid media, Rappaport-Vassilliadis Soy - RVS (Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna, Brescia, Italy) and Muller Kaufmann Tetrathionate Novobiocin - MKTTn (Oxoid Deutschiand GmbH, Wesel, Germany) were inoculated with 100 µl and 1.0 ml of culture broth respectively and incubated at 41.5°C and 37°C for 24±3 hours, respectively. In duplicate, a loop-ful of broth was streaked on Xylose Lysine Desoxycholate - XLD (Oxoid Deutschiand GmbH, Wesel, Germany) and Brillant Green Agar - BGA medium (Meus s.r.l, Piove di Sacco, Padova, Italy) and incubated at 37°C for 24 hours. Colonies referable to *Salmonella*, appear pink with or without a black point in the center of the colony on XLD or BGA medium.

RESULTS

Serological results: From 971 ornamental hens, 240 (24.71%) blood samples (10 from each farm) were taken, 231 (24.01%) were analysed (Table 2). Nine serum samples (9/240, 3.75%) were rejected for insufficient quality. The percentage of animals sampled for each farm, in the age range considered, was between a minimum of 13.16% and a maximum of 71.43% (Table 2). In total, the positive farms were 4/24 (A-B-E1-E5) (16.67%) and the percentages of animals sampled in these farms were 25.64%, 18.87%, 16.67% and 27.78% respectively (Table 2). Based on the results obtained from serological tests in the positive farms (Table 3), the *Salmonella* serotypes detected and their prevalence were: in farm A, 1 sample resulted positive for *S.T.* (10.0% of prevalence); in farm B, 2 samples were positive for both serotypes and 2 samples for *S.E*. The prevalence for *S.T.* was 20.0% while for *S.E.* was 40.0%. In farm E1, 1 sample was positive for *S.E.* (12.5% of prevalence) and 2 samples for *S.T.* (25.0% of prevalence); in farm E5, 2 samples were positive for *S.T.* (25.0% of prevalence) (Table 3). Positive samples were in total 10/231 (4.33%). Out of 10 positive samples, 5/231 (2.16%) were positive for *S.E*., 7/231 (3.03%) for *S.T*., and 2/231 (0.87%) were positive for both serotypes (Table 3). About individual farms examined, 2/24 (8.33%) were positive for *S.E.* (B-E1) and 4/24 (16.67%) for *S.T.* (A-B-E1-E5). No farm resulted positive only for *S.E*. In 2 farms (H-L) *TSA* and *ELISA* serological test for *S.T.* had provided inconclusive results and the samples were excluded from the total count of the positive samples. In farms B and E1, positive for both serotypes, the prevalence was 40.0% and 12.5% for *S.E*., and 20.0% and 25.0% for *S.T.* respectively (Table 3).

Microbiological results: Microbiological analysis performed on 24 yolk pools and 24 shell pools were negative, with a *Salmonella* isolation rate of 0.0% (0/48).

Statistical analysis: Let's consider the - farms where there are infected animals and that the spread rate is higher than 5% as predetermined (spread rate over the total population). Using the *Cannon&Roe* formula, the disease spread rate within a group can be detected with 95% confidence level depending on sample and group size. For example, by taking 10 blood samples from a farm represented by 40 hens, there is a 95% probability of detecting the presence of *S.E.* and *S.T.* in the group, if the spread rate is equal to or higher than 22%. In our study, out of 24 farms we have a rate of 15% in the farm with the lowest number of hens (farm E7, 14 hens) and a rate of 24% in the farm with the highest number of hens (I1, 76 hens) (Table 2).Therefore, the collection of 10 blood samples is sufficient to detect the presence of *S.E.* and *S.T.*, with 95% confidence level. In 4 positive farms (Table 3) the spread rate is 25%. It seems appropriate to use 10 blood samples as the number of samples to be taken, as it allows to find, with 95% confidence level, at least one infected subject. Paradoxically, in 2 farms out of 4 tested positive, positivity for *S.E.* and *S.T.* was found even with a number of samples analysed below 10 (farm E1 8/10; farm E5 8/10) (Table 3).

DISCUSSION

The present study detected 4/24 positive farms (16.67%), with a total of 10/231 samples positive for *Salmonella* spp. In particular, 2.16% for *S.E.* and 3.03% for *S.T.*, with 2/231 (0.87%) samples positive for both serotypes, unlike was found in the study conducted by Brown *et al.* (2018) which detected a 0.0% serum prevalence for *S.E.* in a small flock of 41 backyard chickens. Brown *et al.* (2018) supports the extreme importance of the size of the sample, in order to obtain a valid and reliable epidemiological data. Our serological positivity, related to the size of the sample (231 blood samples), confirm that it is probably essential to increase the size of the sample when serological and epidemiological investigations are carried out, apart from what established by the preliminary statistical analysis. As a matter of fact if fewer animals would had been sampled, as 58 chickens, we would have probably underestimated the serological positivity, declaring farms as false-negative, when they were not. All pools of yolk and shell were negative for *Salmonella* spp. In culture method (0.0%). Is known that *Salmonella* spp. colonize the reproductive tissues, ovaries and oviducts, and it can survive inside the egg as well, in particular *S.E.* (Guard-Petter, 2001). Moreover, *Salmonella* spp. survives in the chicken endothelia reticulum, which has been demonstrated as an important host specificity, explaining their potential isolation in eggs (Foley *et al*., 2013). For these reasons, poultry can be persistent subclinical shedders, they appear healthy but can intermittently shed

bacteria and considering alternates eliminatory phases and latency phases (Behravesh *et al*., 2014), and become a possible reservoir of *Salmonella*. The negative result of isolation could be related with these factors. It should also be considered that the poultry investigated are not subject to particular productive and reproductive stress, opposed to intensive breeding. The negative result of the isolation of *Salmonella* from eggs could be also explained by the productive factors of the hens and correlated by age, in addition to the latency. Pure breeds produce less eggs than industrial laying hens. In our opinion, the number of eggs produced, a very variable age range of hens and production inconstancy, could affect the elimination of *Salmonella* in eggs. It must also be considered that backyard poultry owners have also limited access to specialized veterinarians and do not have the habit to investigate the causes of sudden death of their animals, which could result in a failure to detect a potential outbreak of *Salmonella* in early stages and consequently a failing in arresting the infection. Therefore, the negative results of the culture tests did not allow to investigate the genetic characterization of the isolates and verify their sensitivity to antibiotics. For further diagnostic investigation, to confirm the positivity of 10 hens, we could not obtain fecal samples and cloacal swabs, because farmers refused an additional assessment, probably fearing serious repercussions on their group of animals. After all, farmers are not subject to a legal obligation to conferred samples for official controls. However, we can state that the positive hens came into contact with *S.E.* and *S.T.* during their life, positivity had been confirmed also by *ELISA* test, and the present study suggest that *S.E.* and *S.T.* serotypes are the potential source of subclinical salmonellosis in ornamental poultry and the circulation of the pathogen raises a potential public health problem. The public health risk of foodborne infections, could increase with the trade of eggs produced by these hens, as permitted by Italian legislation. Human salmonellosis is most often of foodborne origin, but other routes of infection, such as contact with live animals and environmental transmission, have also been identified (Baker *et al*., 2007; O' Reilly *et al*., 2007). These animals are considered like a pet (McDonagh *et al.,* 2018), and may inadvertently increase the risk of disease transmission, such as with only direct contact with feathers or beaks (Nichols *et al*., 2018). Based on the anamnestic data obtained in this study, the serum positivity for S.E. and S.T. found in the tested farms has not been associated with outbreaks of *Salmonella* infection of foodborne origin or linked to direct contact with live animals. The flocks examined were similar for zootechnical characteristics (free-range farms), chicken breeds reared (Mediterranean light chickens, Asiatic, Polish, French and American breeds) and presence of other ornamental avian species. The high serological prevalence for both serotypes of *Salmonella* spp. observed in two flocks with 12.5% and 40.0% for *S.E.*, and 20.0% and 25.0% for *S.T.* respectively in B and E1 farm, compared with others 22 farms, could be explained by the higher chicken turnover in these two farms, a recent infection or recent participation to beauty competitions, where poultry come into contact with other subjects or the possibility of multiple interactions with other species. It should not be excluded exchanges of infected eggs between farmers may also take place. The presence of other avian ornamental species in these farms, conditions of biosecurity often not implemented (breeding of different species in separate paddock), general farm management, age and species of birds and exposure to infected birds/environments could have contributed to the observed difference in seroprevalence levels. The other avian species such as peacocks, pheasants, geese and ducks, have different reproductive cycles, oviposition does not occur in December or January, so it was not possible to analyze the eggs of these animals. Moreover, due to the excessive stress caused by the capture, it was decided to exclude these species from serological tests, as further sampling. Remains to investigate the role of these other avian species in the spread and maintenance of *Salmonella* spp. on farms. To our knowledge, this is the first investigation of seroprevalence for *S.E.* and *S.T.* associated to culture method on eggs in ornamental hens in Italy, and this study provides interesting preliminary information regarding the current prevalence of *Salmonella* in these types of farms. Anyway, raises many more questions regarding how this information fits in Italy current surveillance and monitoring of the disease, due the lacking about specific legislation. Due to our findings, we believe it is necessary to investigate further the circulation of zoonotic *Salmonella* species in these types of farms using a microbiological and biomolecular test, in order to have a clear view of its prevalence in backyard chicken flocks. Biosecurity practices of small poultry keepers are poor compared to commercial industries. Backyard chickens often have a regular contact with wild birds or mammals, or are often moved in promiscuous environment, for example for beauty competitions. We must also consider that the environmental persistence of *Salmonella* spp., high turnover of chickens and assiduous participation in beauty competitions, pose significant barriers to its elimination in the farms.

Conclusions: This study confirm that *S.E.* and *S.T.* are widespread in studied poultry farms as asymptomatic form. For this reason, it could be useful to inform farmers that a regular and periodic control of animals and eggs or meat that they consume or sell and limit direct contact with poultry (McDonagh *et al*., 2018), is very important to prevent and limit the spread of *Salmonella* foodborne infections, despite the inconsistency of current Italian legislation. Furthermore, the application of biosecurity standards is clear and simple for industrial breeders, but it is not so obvious that backyard farmers may be aware of the steps required to keep infectious diseases out of their flock and prevent their spread.

Authors contribution: AG and MF, conceived and designed the study. AG, GT and GM executed the experiments and PR and ER analysed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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