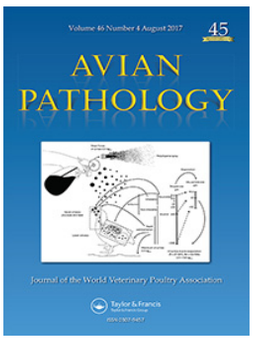

Avian Pathology

ISSN: 0307-9457 (Print) 1465-3338 (Online) Journal homepage: https://www.tandfonline.com/loi/cavp20

Further investigations on the epidemiology of fowl typhoid in Brazil

Anny Lucia del Pilar Celis-Estupiñan, Diego Felipe Alves Batista, Marita Vedovelli Cardozo, Andrei Itajahy Secundo de Souza, Lucas Bocchini Rodrigues Alves, Adriana Maria de Almeida, Paul Andrew Barrow, Angelo Berchieri Jr. & Oliveiro Caetano de Freitas Neto

To cite this article: Anny Lucia del Pilar Celis-Estupiñan, Diego Felipe Alves Batista, Marita Vedovelli Cardozo, Andrei Itajahy Secundo de Souza, Lucas Bocchini Rodrigues Alves, Adriana Maria de Almeida, Paul Andrew Barrow, Angelo Berchieri Jr. & Oliveiro Caetano de Freitas Neto (2017) Further investigations on the epidemiology of fowl typhoid in Brazil, Avian Pathology, 46:4, 416-425, DOI: 10.1080/03079457.2017.1299922

To link to this article: https://doi.org/10.1080/03079457.2017.1299922

AVIAN PATHOLOGY, 2017
VOL. 46, NO. 4, 416–425
https://doi.org/10.1080/03079457.2017.1299922

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Further investigations on the epidemiology of fowl typhoid in Brazil

Anny Lucia del Pilar Celis-Estupiñan[a], Diego Felipe Alves Batista[a], Marita Vedovelli Cardozo[a], Andrei Itajahy Secundo de Souza[a], Lucas Bocchini Rodrigues Alves[a], Adriana Maria de Almeida[a], Paul Andrew Barrow[b], Angelo Berchieri Jr.[a] and Oliveiro Caetano de Freitas Neto[a,c]

[a]Department of Veterinary Pathology from the School of Agricultural and Veterinarian Sciences, Universidade Estadual Paulista (FCAV/Unesp), São Paulo, Brazil; [b]School of Veterinary Medicine and Science, The University of Nottingham, Loughborough, UK; [c]Department of Veterinary Sciences, Federal University of Paraiba, Paraíba, Brazil

ABSTRACT

Salmonella Gallinarum (SG) causes fowl typhoid (FT), a disease responsible for economic losses to the poultry industry worldwide. FT has been considered to be under control in Brazil; nevertheless, since 2012 it has frequently been identified in poultry farming of several Brazilian states. The present study was aimed at assessing (i) the pathogenicity of a SG strain recently isolated from an FT outbreak affecting chickens of both white and brown layers; (ii) the transmission of SG through eggs and hatching; (iii) the effects of antibiotic therapy on SG persistence in poultry tissues and on its vertical transmission and (iv) the genetic profiles of strains isolated over 27 years by Pulsed Field Gel Electrophoresis. Clinical signs, mortality and gross pathologies were very marked amongst brown-egg layers. In contrast, clinical manifestation of FT and mortality were barely present amongst the white-egg layers, although bacteria could be re-isolated from their tissues up to 35 days after infection. No bacteria were re-isolated from the laid eggs, so vertical transmission was not achieved, although newly hatched uninfected chicks became infected spontaneously after hatching. Antibiotic therapy was shown to be effective at reducing mortality, but was not able to clear infection or to favour SG transmission via eggs. Our pulsed field gel electrophoresis results revealed an endemic SG clone that may have been circulating in the Brazilian poultry flocks in the south and southeast regions for more than 20 years. The results suggest that the industrial incubation of SG-contaminated eggs could be one of the factors responsible for the spread of FT in Brazil.

ARTICLE HISTORY
Received 1 September 2016
Accepted 21 February 2017

KEYWORDS
Salmonella Gallinarum; hatchery; transmission; PFGE; chicken lines

Introduction

Salmonella enterica subsp. *enterica* serovar Gallinarum biovar Gallinarum (SG) causes fowl typhoid (FT), a severe systemic disease observed mainly in adult birds (Shivaprasad & Barrow, 2013). FT induces significant economic losses by diminishing egg production and causing high mortality, which may reach 80% of affected flocks (Shivaprasad, 2000; Barrow & Freitas Neto, 2011). The commonest clinical signs consist of sudden decrease in feed and water consumption, somnolence, fever, ruffled feathers and greenish-yellow diarrhoea, and death usually occurs between 4 and 10 days after infection (Pomeroy, 1978; Shivaprasad & Barrow, 2013). Typical gross pathologies in the acute phase of infection include swollen, greenish to bronze livers, enlarged spleens and increased pericardial fluid volume with turbidity, and more chronic disease presents, in addition, large necrotic areas of the myocardium (Pomeroy, 1978; Ezema *et al.*, 2009; Shivaprasad & Barrow, 2013).

Chickens are considered to be the main natural host for SG, but susceptibility to FT amongst lineages is variable. Oliveira *et al.* (2005) and Freitas Neto *et al.* (2007) demonstrated that the light lineages reared in Brazil were resistant to FT, whereas both heavy and semi-heavy lineages were susceptible. The genetic resistance to systemic salmonellosis in chickens depends on a number of factors, and amongst them, the Slc11a1 (previously *Nramp1*, Hu *et al.*, 1997) and *SAL1* (Mariani *et al.*, 1998). These genes are known to increase macrophage activity against intracellular *Salmonella* (Wigley *et al.*, 2002, 2006). Thus, chickens belonging to resistant lineages show only moderate pathology and low mortality rates (Oliveira *et al.*, 2005; Freitas Neto *et al.*, 2007). Despite the ability to withstand clinical disease, such birds may harbour SG for long periods in their tissues and become key players as a source of infection within the poultry flocks (Berchieri Jr *et al.*, 2001).

Experimental data on the epidemiology of FT have shown that SG infection results in either clinical disease or bacterial clearance from tissues (Berchieri Jr *et al.*, 2001; Oliveira *et al.*, 2005). Therefore, persistent infection and vertical transmission that occur with

CONTACT Oliveiro Caetano de Freitas Neto oliveirocaetano@yahoo.com.br
Supplemental data for this article can be accessed here: http://dx.doi.org/10.1080/03079457.2017.1299922

pullorum disease seem unlikely to occur in the same way, suggesting that its infection biology is very different from this taxonomically closely related serovar (Berchieri Jr *et al.*, 2001). Despite this, pioneering studies indicated the possibility of SG being transmitted both vertically and horizontally (Beach & Davis, 1927; Beaudette, 1930; Hall *et al.*, 1949). Experimental attempts to reproduce vertical transmission in chickens, however, did not succeed and only horizontal transmission was achieved, with no SG being recovered from laid eggs (Berchieri *et al.*, 2000; Oliveira *et al.*, 2005).

FT control is based on pathogen identification and slaughter of infected birds (Barrow & Freitas Neto, 2011). These measures are considered the basis of FT control in many European and North American countries (Shivaprasad & Barrow, 2013). However, antimicrobial therapy is still used in some countries to treat FT (Nonga *et al.*, 2010; Dutta *et al.*, 2015). A variety of chemotherapeutic agents have been found to be effective at reducing mortality, but they do not eliminate infection from a flock, since birds remain infected after treatment and can also be reinfected from the local environment (Moore, 1948; Wilson, 1956; Gordon & Tucker, 1957). Furthermore, this practice has led to increased resistance to fluoroquinolones in some countries (Smith *et al.*, 1981; Lee *et al.*, 2003, 2004). In Brazil, the use of fluoroquinolones to treat flocks with FT is also a common practice in egg-producing farms (Penha Filho *et al.*, 2016). Multi-resistance has been found in strains from Korea (Lee *et al.*, 2003; Kang *et al.*, 2010).

According to the World Organization for Animal Health (OIE), FT remains a problem for the poultry industry in many countries in South and Central America, Asia and Central Africa (OIE, 2015). In the last decade, sporadic outbreaks of FT have been reported every year in Brazil. In 2012, 14 outbreaks of FT were reported to the OIE and since then FT has been identified in several poultry farms across the country, resulting in the status of "disease present but without quantitative data" on the OIE database (OIE, 2015).

The causes for FT re-emergence and spread in Brazilian poultry flocks remain unclear and the following hypotheses have been formulated: (i) commercial lines of chickens have improved genetically to be resistant to FT and do not develop clinical disease. However, it is possible that they can become persistently infected, favouring the transmission and maintenance of SG in poultry farms; (ii) chemotherapy in outbreaks of FT may have been adopted in breeding flocks, facilitating vertical/hatchery transmission of SG; (iii) SG-contaminated eggs are reaching the hatcheries, favouring FT transmission to newly hatched chickens and (iv) a distinct SG strain presenting new pathogenicity features may have arisen and spread in the Brazilian flocks.

The present study was devoted to testing all the hypotheses listed above, aiming at (a) evaluating the pathogenicity of a SG strain isolated in 2012 from an outbreak of FT using commercial lines of laying hens currently used in Brazil; (b) assessing whether the use of antibiotic therapy in diseased birds could favour vertical transmission of SG; (c) verifying the possibility of vertical/hatchery transmission by a series of experiments on egg contamination followed by incubation and (d) analysing comparatively the genetic profiles of SG strains, isolated over a span of 27 years from different Brazilian states, using pulsed field gel electrophoresis (PFGE).

Materials and methods

Strain and growth conditions

SG strain 1035 was isolated in 2012 from a case of FT presented at the Laboratory of Avian Diseases of the School of Agricultural and Veterinary Sciences, FCAV/Unesp. For ease of enumeration, a spontaneous nalidixic acid-resistant mutant (SG 1035 Nal^r) was prepared and stored at −80°C in lysogeny broth (Invitrogen, Grand Island, NY, USA – Ref. 12795–027) supplemented with 30% glycerol (MERCK Darmstadt, Germany – Ref. k30402394). SG 1035 Nal^r was used in all *in vivo* assays (Experiments 1–6) and bacterial cultures were prepared in lysogeny broth as described previously by Berchieri *et al.* (2001).

In Experiment 7, 43 strains of SG were used (Supplementary Table 1). Field strains were isolated from different farms in São Paulo, Rio Grande do Sul, Minas Gerais and Mato Grosso states at different periods. These strains were identified by serotyping and biochemical tests at the National Agriculture and Livestock Laboratory or Oswaldo Cruz Foundation, which are official laboratories associated with the Brazilian Ministry of Agriculture, Livestock and Supply. ATCC 9184, SG vaccine 9R and SG 1035 Nal^r strains were included as references. All bacteria used in this study were maintained at −80°C in the Laboratory of Avian Diseases (FCAV/Unesp).

Chickens and fertilized eggs

Chickens from distinct lines of commercial laying hens, belonging to semi-heavy (brown) and light (white) varieties, were used in Experiments 1 and 2, whereas heavy broiler breeders were tested in Experiment 3. These birds were reared in controlled temperature rooms, within cages with wire floors, and received *ad libitum* feed and water. Inoculation of fertilized eggs was divided into three experiments as follows: Experiment 4 – inoculation of eggs from both semi-heavy (brown) and light (white) breeders; Experiment 5 – inoculation of eggs from broiler breeders; and

Experiment 6 – inoculation of eggs from grandparent hens. All experiments were performed in the bird facilities of the Laboratory of Avian Diseases (FCAV/Unesp) in accordance with institutional guidelines and had been approved by the institutional internal Ethical Committee (Approval number 04233/14. Date of approval: 2 April 2014).

Experiment 1: susceptibility of commercial laying hen lines to FT

One hundred and fifty one-day-old chickens from three lines of commercial brown layers (groups A, B and C) and 150 one-day-old chickens from three lines of commercial white laying hens (groups D, E and F) were used. On arrival, the chickens were submitted to cloacal swabbing to exclude infection with *Salmonella* spp. and then distributed randomly into 12 groups as shown in Table 1.

At 27 days of age, 6×10^8 colony-forming units (CFU) in 0.5 ml of SG 1035 Nal^r was administered into the crops of the birds of groups A_1–F_1 by gavage needles. Meanwhile, chicks of groups A_2–F_2 received 6×10^6 CFU SG 1035 Nal^r as described above. The birds were observed for four weeks and clinical signs and mortality were recorded daily. A proportion of the surviving birds were euthanized at 28 days post-inoculation (dpi) and the remainder at 35 dpi. On these days, samples from livers, spleens and ovaries were collected aseptically during post-mortem examination for bacterial isolation. These samples were processed as described by Berchieri *et al.* (2001).

Experiment 2: infection of laying hens and fluoroquinolone therapy

Thirty 50-week-old commercial laying hens, of white and brown varieties, were inoculated orally into the crop with 2×10^9 CFU in 2 ml of SG 1035 Nal^r using gavage needles. Brown birds were treated from 3 to 8 dpi by adding 10% enrofloxacin (100 Enrotec FATEC SA, Arujá, Brazil) to the water at a concentration of 20 mg/kg body weight (bw). At 44 dpi, the remaining chickens were euthanized and samples of livers, spleens and ovaries collected for bacteriological examination. The eggs laid during the experiment were collected aseptically and processed for bacterial isolation as follows: the contents from five eggs were poured out into a sterile plastic container and mixed together; their shells were placed into a second sterile plastic container and macerated. Then, the plastic containers were filled with 200 ml of selenite broth (OXOID®, Basingstoke, UK, Ref. CM0395), containing 4 mg/ml novobiocin (Selenite broth plus novobiocin [SNNov], Sigma-Aldrich, St Louis, MO, USA), and incubated at 37°C for 18 h. After incubation, the cultures were homogenized, streaked onto brilliant green agar, containing 100 µg/ml nalidixic acid, and incubated at 37°C for 18 h. Colonies resembling *Salmonella* colonies on brilliant green agar were recorded as positive.

Experiment 3: infection of broiler breeders followed by antibiotic treatment

Twenty 36-week-old broiler breeders, previously vaccinated at 12 weeks of life with a commercial inactivated *Salmonella enteritidis* vaccine, were used. The chickens were divided into two groups based on the infectious dose and antibiotic therapy adopted. Group G birds were inoculated orally, using oral gavage, with 2×10^4 CFU in 3 ml of SG 1035 Nal^r, whereas group H birds received 2×10^6 CFU inoculum in 3 ml. Twenty-five per cent kanamycin sulphate (Kanainjecto 250 – FATEC SA) (0.2 ml/kg bw) was injected daily between 6 and 12 dpi into the breast muscle of group G birds. Meanwhile, water containing 10% enrofloxacin (100 Enrotec FATEC SA) was provided to group H chickens (20 mg/kg bw), from 3 to 5 dpi, and from 6 to 12 dpi these birds received the same treatment applied to group G birds. Laid eggs were collected aseptically and cultured for bacteriological evaluation as described for Experiment 2. The birds were necropsied at 29 dpi for bacteriological examination (Berchieri *et al.*, 2001).

Experiment 4: incubation of fertilized eggs from brown and white breeder laying hens

A total of 90 fertilized eggs from both brown and white varieties of breeder laying hens were used. Egg shells were disinfected on the air cell region with 70% ethanol and divided into four groups as follows: infected white (group I) and brown (group J) eggs, and non-infected white (group K) and brown (group L) eggs. Using a sterile metallic mandrel, a 0.5-mm diameter hole was made on the egg surface, at the air cell region, and 6×10^8 CFU in 0.1 ml of SG Nal^r 1035 were inoculated gently into the yolk using a disposable 1-ml syringe and a 25×7 mm needle. Non-infected eggs (negative controls) were inoculated with 0.1 ml of sterile phosphate-buffered saline (1x PBS, pH 7.4). The holes in the egg shells were sealed with candle wax.

Eggs from all groups were incubated for 21 days in the same single-stage incubator (CP 100 Premium Ecológica, Belo Horizonte, MG, Brazil). Over the first 18 days, the temperature was maintained at 37.5°C, but switched to 38°C during the last three days of incubation. Humidity was kept between 28% and 30% throughout the experiment. At 21 dpi, the unhatched eggs were opened and then submitted to bacteriological analyses. They were placed individually into sterile plastic containers (shell and content), macerated and then enriched with 200 ml SNNov. After brief homogenization, the enriched samples were incubated at 37°C for 18 h. The newborn chicks, in turn, were

Table 1. Mortality rate of birds of the brown and white laying hen lines infected with SG Nal^r 1035.

			Days post SG inoculation																		
			2	3	4	5	6	7	8	9	10	11	14	15	16	17	19	23	24	Mortality[a]	
Line	Group	Number of birds	Number of dead birds by day																	Total	%
Brown (A, B, C)	A_1	26	–	–	1	5	12	5	2	–	–	–	–	–	–	–	–	–	–	25	96^A
	B_1	25	1	1	1	6	13	1	1	–	–	–	–	–	–	–	–	–	–	24	96^A
	C_1	23	–	1	1	11	7	2	1	–	–	–	–	–	–	–	–	–	–	23	100^A
	A_2	30	–	1	1	2	11	6	5	–	1	–	1	–	–	–	–	1	–	29	96^A
	B_2	25	–	–	–	2	6	3	5	2	–	1	–	–	1	1	1	–	1	23	92^A
	C_2	25	–	–	–	2	10	6	2	–	–	–	–	1	–	–	–	2	–	23	92^A
White (D, E, F)	D_1	27	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0^B
	E_1	24	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	4^B
	F_1	25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0^B
	D_2	25	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	4^B
	E_2	25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0^B
	F_2	25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0^B

Note: A, B and C: lines of brown-egg laying hens (semi-heavy)/D, E and F: lines of white-egg laying hens (light)/Groups A_1–F_1 were challenged with 6×10^8 CFU SG Nal^r 1035, whilst groups A_2–F_2 received 6×10^6 CFU.

[a]Mortality pair-wise comparison: different uppercase letters in the same column indicates that mortality rates are statistically different between the compared groups by chi-square test (χ^2, $P < 0.01$).

distributed in separated boxes according to the experimental group and observed for over 10 days before being euthanized for microbiological analyses.

Experiment 5: incubation of fertilized eggs from heavy broiler breeders

In total, 90 fertilized eggs from a 39-week-old heavy broiler breeder flock, which had been previously vaccinated at 12 and 21 weeks of life with a commercial inactivated *S. enteritidis* vaccine, were used. Eggs were divided into two groups and procedures of infection, incubation and microbiological analysis were performed as described for Experiment 4. Group M eggs were infected with 6×10^8 CFU in 0.1 ml of SG 1035 Nal^r inoculated into the yolk, whilst those from group N were kept as uninfected controls. Monitoring procedures were those already described for Experiment 4.

Experiment 6: incubation of fertilized eggs from broiler grandparents

A total of 90 eggs from a 32-week-old grandparent flock were used. They were divided into three groups as follows: group O eggs were inoculated via the air cell with 5×10^6 CFU in 50 µl of SG 1035 Nal^r; group P eggs were cracked gently and 50 µl of inoculum containing 5×10^7 CFU SG 1035 Nal^r were spread over the cracked region and group Q eggs were maintained as uninfected controls. The surfaces of group P eggs were allowed to dry before being placed in the incubator. Incubation and monitoring procedures of hatched chicks and microbiological analysis were performed as described for Experiment 4.

Experiment 7: genotyping of Brazilian SG strains by PFGE

SG isolates were genotyped by using the standard protocol of PulseNet PFGE, described by Ribot *et al.* (2006). PFGE was performed using the CHEF-DR® III variable angle system (Bio-Rad, Hercules, CA, USA) set up as follows: initial switch time value of 2.2 s, final switch time of 54.2 s at a gradient of 6 V/cm and an included angle of 120°. One per cent Agarose Certified Pulsefield (Bio-Rad) gels were electrophoresed for 21 h in 0.5x Tris-borate-EDTA buffer at 14°C. Bacterial DNA was digested with *Xba*I (Sigma-Aldrich) and *Salmonella* Braenderup strain H9812 was used as standard (ladder) for the method. After electrophoresis, the gels were stained with 0.5 µg/ml ethidium bromide solution and visualized by UV light.

Data analysis

Mortality rates in Experiment 1 were analysed by a non-parametric chi-square test (Greenwood & Nikulin, 1996), considering a 5% significance level ($P < 0.01$). The number of infected livers, spleens and ovaries over the 35-day Experiment 1 was recorded and values compared between groups using Fisher's exact test, considering a 5% significance level ($P < 0.05$). Similarities based on the PFGE types were calculated using the Dice coefficient with 1% tolerance and 0.5% optimization. The Unweighted Pair Group with Arithmetic Mean method was applied to the output matrix in order to construct a dendrogram by means of the Bionumerics software version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium).

Results

Experiment 1: susceptibility of different egg-layer lines to FT

Three days post-infection (dpi), birds from groups A_1, B_1, C_1, A_2, B_2 and C_2 (brown layer lines) developed the following clinical signs: depression, weakness, loss of appetite, ruffled feathers, dropped wings and closed eyes. In contrast, chickens from groups D_1, E_1, F_1,

D_2, E_2 and F_2 (white layer lines) showed no clinical signs. The mortality rate was significantly higher amongst brown layer than amongst white-laying hens ($P < 0.05$) (Table 1). In total, only two white birds (one from group E_1 and another from D_2) died over the experiment, but without apparent clinical signs. The peak of mortality was reached between 5 and 8 dpi. No correlation between the infectious dose and mortality rates was found in this study ($P > 0.05$). Furthermore, differences in mortality amongst varieties of the same line (either semi-heavy or light) were not statistically significant ($P > 0.05$). However, in groups A_2, B_2 and C_2 (semi-heavy lines) some deaths occurred much later in the experiment, between 10 and 24 dpi. SG was recovered from the spleens and livers of chickens of all lines at 28 and 35 dpi, with the exception of birds in groups B_2 and C_2 (Table 2).

Experiment 2: infection of laying hens and fluoroquinolone therapy

Two days after infection, the brown birds began to show typical clinical signs of FT observed in Experiment 1. Amongst the white birds, these clinical signs were milder and appeared at 5 dpi. Thus, enrofloxacin treatment was carried out only in brown birds (3–8 dpi) and clinical signs ceased between 9 and 15 dpi, appearing again six days later.

Egg production was diminished for both brown and white hens after infection (Table 3). Brown birds completely stopped producing eggs during the first week after infection, whilst white birds progressively decreased oviposition, which fell down to 2% one week after inoculation. Egg production was restored only to 50% in both varieties of chickens even by the end of the experiment (5 weeks after infection). In total, 280 eggs were collected, but none of them was positive for SG.

Seven birds of the brown variety died between 4 and 5 dpi and the remaining birds were visibly sick. Mortality ceased during the antibiotic treatment period, but it returned six days after chemotherapy completion. Four more birds from the brown lines died between 16 and 18 dpi, totalling 11 chickens. In contrast, only one white bird died (6 dpi) during the experiment. The remainder white (24/25) and brown (14/25) hens were euthanized at 44 dpi. SG was then recovered from two spleens, two livers and four ovaries from the white chickens, but not from the organs of the brown birds. Severe gross pathology such as pericarditis, hepatosplenomegaly, white spots on livers and spleens, and haemorrhagic follicles were observed in the brown chickens at post-mortem examination. There was no evident gross pathology in white birds.

Table 2. SG persistence in livers and spleens of the surviving chickens from Experiment 1 during late infection (28 and 35 dpi).

Line	Group	Total birds tested at 28 dpi	Total positives[a]	Total birds tested at 35 dpi[b]	Total positives[a]
Brown (A, B, C)	A_1	1	1^A	–	–
	B_1	1	1^A	–	–
	C_1	–[c]	–[A]	–	–
	A_2	1	1^A	–	–
	B_2	2	0^A	–	–
	C_2	2	0^A	–	–
White (D, E, F)	D_1	17	3^A	10	1^A
	E_1	13	4^A	10	3^A
	F_1	14	11^B	10	6^B
	D_2	14	7^A	10	3^A
	E_2	15	4^A	10	1^A
	F_2	15	6^A	10	4^A

Note: A, B and C: lines of brown-egg laying hens/D, E and F: lines of light white-egg laying hens/Groups A_1–F_1 were challenged with 6×10^8 CFU SG Nal^r 1035, whilst groups from A_2 to F_2 received 6×10^6 CFU.

[a]Comparative analyses were performed between the groups within a line (brown or white) per day of sampling (28 or 35 dpi). Thus, different uppercase letters in the same column indicates that mortality rates are statistically different by chi-square test (χ^2, $P < 0.01$).

[b]The few surviving brown line birds were all tested at 28 dpi so that only white line chickens were tested at both 28 and 35 dpi.

[c]There was no bird to test since mortality was achieved for 100% of the chickens at 8 dpi (Table 1).

Experiment 3: infection of broiler breeders treated with antibiotics

Eggs produced during this experiment were cultured, but SG was not isolated. Birds treated with enrofloxacin and kanamycin after infection showed only loss of appetite and diarrhoea. In contrast, birds that received only kanamycin developed additional clinical signs such as ruffled feathers, pale heads and shrunken combs. In total, four birds died between 5 and 7 dpi, one treated with both antibiotics used in this study and three that had been treated only with kanamycin. Surviving hens were euthanized at 29 dpi and post-mortem examination revealed gross pathologies such as hepatosplenomegaly, congested and friable livers and haemorrhagic or atrophic ovaries. SG was isolated from the ovary of a bird that had been treated with both kanamycin and enrofloxacin.

Experiment 4: incubation of fertilized eggs from brown and white breeder laying hens

At 21 days of incubation, eight chicks hatched from the uninfected groups (three of group K [uninfected white]

Table 3. Egg production before and after experimental infection with SG 1035 Nal^r.

Weeks before and after challenge[a]	Egg production (%)[b] Brown layers	White layers
−2	86	84
−1	79	82
0	40	45
1	0	2
2	0	14
3	6	48
4	11	47
5	14	53

[a]Antibiotic treatment was carried out during weeks 0 and 1 after challenge.

[b]Number of eggs produced by chickens (%).

and five of group L [uninfected brown]), but no hatching was observed amongst the infected eggs. The unhatched eggs were then opened and examined (Table 4). SG was recovered from the eggs of the infected groups only (groups I [white] and J [brown]). Newborn chicks were housed separately, complying with the experimental groups, and monitored daily. Two birds of groups K and L died suddenly soon after hatch, but *Salmonella* spp. were not isolated from their organs (liver and spleen). During the second week after hatching, two birds of group K (white) and two of group L (brown) died. SG was isolated from their livers and spleens, and gross pathologies such as pericarditis and hepatosplenomegaly were observed. The remaining chicks were euthanized on day 10 post-hatching and cultures from their livers and spleens were positive for SG.

Experiment 5: incubation of fertilized eggs from heavy broiler breeders

At 21 days of incubation, 32 chicks hatched from group N eggs (uninfected control). Unhatched eggs of infected (group M) and uninfected groups were opened and examined (Table 4). SG was re-isolated from all group M eggs examined. In contrast, group N eggs were negative for SG. From 3 to 10 days after hatching, eight chicks died and SG was recovered from their livers and spleens. The remaining birds were euthanized at 10 dpi, and their livers and spleens were also SG positive.

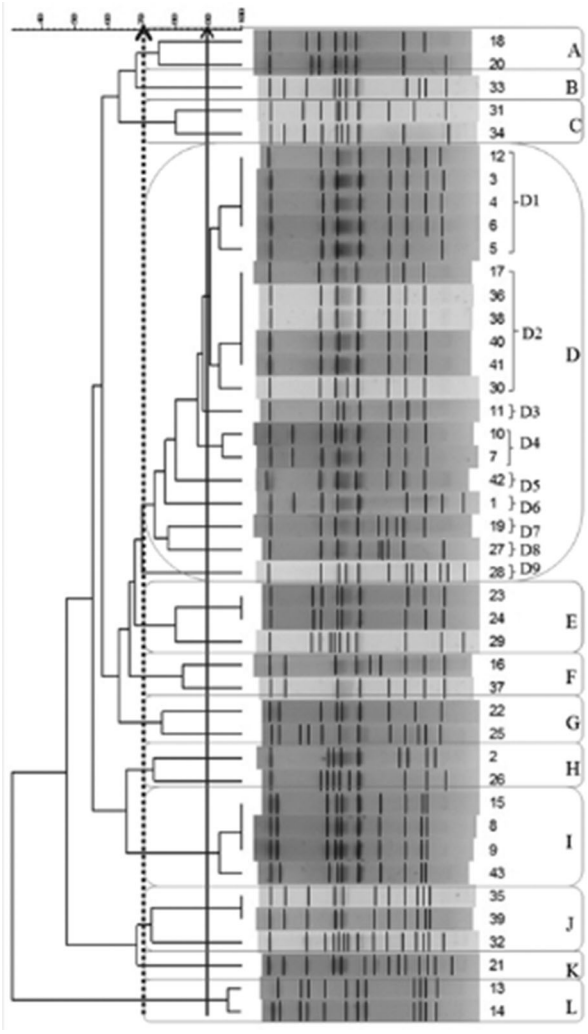

Figure 1. Dendrogram clustering the 43 SG strains genotyped by PFGE in this study. The broken line cuts off the dendrogram at 70% similarity (profiles A–L) and the continuous line cuts off the dendrogram at 90% similarity (sub-profiles D1–D9).

Experiment 6: infection of fertilized eggs from grandparents

At 21 days of incubation, 35 chicks hatched: 17 from group O (inoculated via the air cell), 11 from group P (inoculated on the shell-cracked surface) and seven from group Q (uninfected control). These newly hatched birds were raised separately until 10 days after hatching. The remaining unhatched eggs were opened, examined and only those from groups O and P were positive for SG. Between 3 and 10 days after hatching, a mortality rate of 64.7% (11/17 chicks) was observed amongst group O chicks, and their livers and spleens were positive for SG. There was no mortality amongst birds in group P and only one group Q bird died soon after birth, but bacteriological analysis showed that organs (liver and spleen) were negative for SG. The remaining chickens were euthanized at 10 days post-hatching and SG was cultured from 100% (6/6) and 54.5% (6/11) of groups O and P chicks, respectively. Furthermore, organs from the six remaining group Q birds were also SG positive 10 days post-hatching. The main results are summarized in Table 4.

Experiment 7: genotyping of Brazilian SG strains by PFGE

The genetic profiles of the 43 SG strains produced by PFGE were clustered in a dendrogram (Figure 1). The pulse-types (genotypes) generated were grouped in 12 profiles (A–L), exhibiting $\geq 70\%$ similarity. Amongst them, profile D showed the most remarkable pattern. Roughly 44% of all tested strains fell into this profile and they were isolated between 1990 and 2014 from farms situated in the southeast and south regions of Brazil. In order to discriminate different clones inside this group, profile D strains were further subdivided into nine sub-profiles (D1–D9) by raising the cut-off line to 90% similarity. Sub-profile D1 included strains isolated in the South Brazil and was closely related to the sub-profile D2, which harboured strains isolated in São Paulo state. Interestingly, strain 17 (sub-profile D2), which was isolated in 1990, was a pulse-type with 100% similarity to some recently isolated stains, including SG 1035 Nal^r (strain 40) used in this study (Figure 1).

Table 4. Summary of the experiments conducted with fertilized eggs. Bacteriological analyses were carried out on unhatched eggs at 21 days of incubation or in the chick organs on day 10 after hatching.

	Experiment 4[a]				Experiment 5[b]		Experiment 6[c]		
	I	J	K	L	M	N	O	P	Q
Total incubated eggs	30	30	15	15	45	45	35	35	20
Number of birds hatched out	0	0	3	5	0	32	17	11	7
SG isolation after incubation (21 days) (%)	100	100	0	0	100	0	100	100	0
Deaths at 1 week after hatching	–	–	1	1	–	6	5	0	1
SG in organs (liver/spleen) (%)	–	–	0	0	–	100	100	0	0
Deaths at 2 weeks after hatching	–	–	2	2	–	2	6	0	0
SG isolation (%)	–	–	100	100	–	100	33	0	0
Euthanized birds	–	–	–	2	–	24	6	11	6
SG isolation (%)	–	–	–	100	–	100	100	100	100

[a]Experiment 4: white line eggs (groups I and K) and brown line eggs (groups J and L). Eggs of groups I and J were inoculated with 6×10^8 CFU SG 1035 Nal^r into the yolk, whereas eggs of groups K and L received PBS.
[b]Experiment 5: Eggs of group M were inoculated in the yolk with 6×10^8 CFU SG Nal^r 1035 and eggs of group N were kept uninfected (negative control).
[c]Experiment 6: Eggs of group O were inoculated with 5×10^6 CFU SG Nal^r 1035 in the air cell and those of group P received 5×10^7 CFU on the surface of the egg shell-cracked surface. Eggs of group Q were kept uninfected (negative control).

Discussion

FT was first described more than a century ago (Klein, 1889). Ever since, a great deal of research activity has been carried out on this disease. As a result, information on immunobiology and epidemiology, in addition to the introduction of effective measures towards controlling FT in commercial poultry flocks, is now available (Barrow & Freitas Neto, 2011; Shivaprasad & Barrow, 2013). Despite this, FT still remains of major economic significance in many parts of the world, including Brazil (OIE, 2015).

The OIE databases show that FT has been reported frequently in Brazilian flocks during recent decades (OIE, 2015), but outbreaks appeared to be generally restricted to flocks of brown laying hens (personal communication). In the last four years, FT appears to have spread out in Brazil and several sectors of the poultry industry are now affected. For instance, not only egg-laying hen flocks, but broilers and even breeder flocks reared in distinct regions have become infected (De Carli *et al.*, 2016). In this study, several factors that could play crucial roles in the SG dissemination were assessed individually under experimental conditions.

It would be possible that a new SG strain, with a distinct genotype and resulting in less clinical disease and showing reduced vertical transmission, has arisen and spread through Brazilian flocks. To test this hypothesis, SG strains isolated from cases and outbreaks of FT from some Brazilian states were genotyped by PFGE. PFGE was used because it is still the gold-standard methodology for bacterial genotyping and has been applied successfully to other epidemiological investigations on FT (Seo *et al.*, 2006; Kwon *et al.*, 2010; Van Immerseel *et al.*, 2013). Our data revealed that the SG strains related to the recently diagnosed FT outbreaks in south (sub-profile D1) and southeast (sub-profile D2) Brazil share a high degree of similarity. It was striking that strain 17 (SG 291/90), isolated in 1990, seems to have become the prevalent genotype in these places, since it shared 100% similarity with strains from São Paulo state, including strain 40 (SG 1035/12) used in this study, and was also closely related to those from South Brazil. The use of PFGE has, therefore, supported the presence of an endemic genotype affecting Brazilian flocks for more than 20 years rather than the emergence of a new genotype.

Genetic improvements in commercial lines of birds might have changed the host–pathogen interaction between SG and chickens, affecting bacterial persistence and vertical transmission. This hypothesis was tested using experimental infection of commercial birds (three lines of brown and three lines of white-laying hens) currently reared in Brazil. The majority of birds belonging to the brown-egg-layer lines presented clinical signs of FT and high mortality, reaching almost 100% between 5 and 8 dpi. Similar results had been reported by Oliveira *et al.* (2005) and Freitas Neto *et al.* (2007) who utilized these brown layer lines reared in Brazil in their experiments (Oliveira *et al.*, 2005; Freitas Neto *et al.*, 2007). From this experiment, no apparent change in the nature of the host–pathogen relationship was observed amongst the brown line hens. In contrast, mortality and clinical signs amongst the three lines of white layer birds were almost absent. Different results were described by Freitas Neto *et al.* (2007) who reported clinical signs of FT and mortality ranging from 7% to 40% amongst white line birds previously reared in Brazil. Apparently, the light laying hens currently used are more resistant to FT than those reared before.

In the present study, approximately 60% of the birds of lines F (white lineage/groups F_1 and F_2) were still infected after 28 dpi, clearly indicating the low efficiency of these birds to clear the bacteria from the tissues. These findings are also in line with the experimental work done by Berchieri Jr *et al.* (2001) using highly inbred White Leghorn lines, where persistence in the tissues for up to 14 weeks was seen in a resistant line (Line N) but not in susceptible lines.

According to Wigley *et al.* (2006), resistance to systemic salmonellosis in birds is expressed at the level of

the macrophage. Macrophages of resistant birds signal more effectively and rapidly and are more able to induce protective Th-1 adaptive responses, which leads to bacterial clearance from tissues in comparison with macrophages from susceptible lines. In spite of that, birds of line F studied here did not seem to clear systemic bacteria as efficiently. These results suggest that birds from light lineages, although apparently healthy, may maintain SG for long periods, acting as a source of infection despite being highly resistant to acute phase morbidity and mortality (Bumstead & Barrow, 1993; Berchieri Jr *et al.*, 2001; Oliveira *et al.*, 2005). This emphasizes the complex nature of the interaction between pathogen and host genetics in determining whether or not persistent infection takes place.

Chemotherapy has been used to treat FT for several decades (Moore, 1948; Wilson, 1956; Gordon & Tucker, 1957). However, this method is not completely satisfactory, since outbreaks may occur after cessation of the therapy. In Brazil, antibiotic therapy is still commonly adopted to treat FT outbreaks in commercial egg-laying hens, but it is not allowed to be used in breeder flocks which must be SG free (Brazil, 2009).

The antibiotic treatment of broiler breeders and brown laying hens, in this study, reduced mortality, but was neither able to completely prevent mortality and morbidity, nor clear SG from the bird tissues. These results are in agreement with previous studies which demonstrated that a variety of chemotherapeutic agents were effective at reducing mortality, but were not able to eliminate infection from a flock because birds remained infected (Moore, 1948; Wilson, 1956; Gordon & Tucker, 1957).

In this study, the antibiotic therapy model applied to Experiments 3 and 4 did not benefit SG towards vertical transmission since SG was not recovered from the laid eggs. A similar observation was also reported by Berchieri Jr *et al.* (2000) and Oliveira *et al.* (2005). Moreover, antibiotic therapy was worthless at preventing reduction in the productive performance, since both white and brown laying hens were unable to reach the expected oviposition potential after infection by SG.

The spread of FT in Brazil suggests the involvement of vertical transmission possibly involving the hatchery as a contributing factor in the dissemination. In order to investigate this hypothesis, SG was inoculated in fertilized eggs of different lines of birds. On day 21 of incubation, SG was still viable and was recovered from the infected eggs regardless of the line studied. Therefore, if SG is able to reach the yolk or air cell during natural infection, or if it contaminates the cracked shell of fertilized eggs, it could spread very readily within the hatchery machine. Although SG-infected embryos were not able to develop, chicks from uninfected eggs became infected after hatching. It is possible that contaminated material, such as shell, albumen, yolk and embryos, that remained in the incubator were the sources of SG. Since the introduction of commercial hatching in the 1910s, incubator transmission became the most important way of spreading pullorum disease (Bullis, 1977). Therefore, although this study is one of the reports that proves the possibility of SG dissemination through egg incubation, it is not new that several control measures, including disinfection, selection and storage of hatching eggs, are crucial to avoid the spread of diseases (Van Roekel *et al.*, 1951; Stuart & Keenum, 1970; Kim & Kim, 2010). The sanitary conditions of the breeder flocks must also be monitored constantly and controlled to avoid infectious diseases that can reach the hatching process and the progeny (Gast, 2013).

According to our results, there is no indication that a new strain of SG, a genetic shift within poultry lines or antibiotic therapy would be responsible for the outbreaks of FT that have happened in the last years in Brazil. Moreover, vertical transmission was not observed. However, SG was still viable in contaminated materials over the incubation and was transmitted to newborn chicks by contact. It is possible that failures in the execution of biosecurity and disease monitoring programmes at different steps (breeding, hatching and production) of the poultry industry could be mainly responsible for the spread of FT in Brazil. New studies might be carried out to assess risk factors such as sampling to detect low numbers of infected birds in the flock, the handling and destination of dead birds and hatchery waste, and the transport for slaughtering of breeder and commercial flocks.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo) (FAPESP) [grant number: 2011/23483-1]; National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico) (CNPq) [grant number: 190778/2013-0] and the Coordination of Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) (CAPES).

References

Barrow, P.A. & Freitas Neto, O.C. (2011). Pullorum disease and fowl typhoid – new thoughts on old diseases: a review. *Avian Pathology*, 40, 1–13.

Beach, J.R. & Davis, D.E. (1927). Acute infection of chicks and chronic infection of the ovaries of hens caused by the fowl-typhoid organism. *Hilgardia*, 2, 411–426.

Beaudette, F.R. (1930). Fowl typhoid and bacillary white diarrhea. *Proceedings of the 11th International Veterinary Congress, London*, 3, 705–723.

Berchieri, A., Jr., Murphy, C.K., Marston, K. & Barrow, P.A. (2001). Observations on the persistence and vertical transmission of *Salmonella enterica* serovars Pullorum and Gallinarum in chickens: effect of bacterial and host genetic background. *Avian Pathology*, 30, 221–231.

Berchieri, A., Jr., Oliviera, G.H., Pinheiro, L.A.S. & Barrow, P.A. (2000). Experimental *Salmonella* Gallinarum infection in light laying hen lines. *Brazilian Journal of Microbiology*, 31, 50–52.

Brazil – Ministry of Agriculture, Livestock and Food Supply. (2009). Normative Instruction n.78 of November 3, 2003. In *Legislation Manual*. National Animal Health Programs in Brazil (pp. 200–207).

Bullis, K.L. (1977). The history of avian medicine in the U.S. II. Pullorum disease and fowl typhoid. *Avian Diseases*, 21, 422–429.

Bumstead, N. & Barrow, P. (1993). Resistance to *Salmonella* Gallinarum, *S. pullorum*, and *S. enteritidis* in inbred lines of chickens. *Avian Diseases*, 37, 189–193.

De Carli, S., Gräf, T., Mayer, F.Q., Cibulski, S., Lehmann, F.K.M., Fonseca, A.S.K., Ikuta, N. & Lunge, V.R. (2016). Draft genome sequence of a *Salmonella enterica* subsp. *enterica* serovar Gallinarum bv. Gallinarum isolate associated with fowl typhoid outbreaks in Brazil. *Genome Announcements*, 4, e00019-16.

Dutta, P., Borah, M.K., Gangil, R. & Singathia, R. (2015). Gross/histopathological impact of *Salmonella* Gallinarum isolated from layer chickens in Jaipur and their antibiogram assay. *International Journal of Advanced Veterinary Science and Technology*, 4, 153–159.

Ezema, W.S., Onuoha, E. & Chah, K.F. (2009). Observations on an outbreak of fowl typhoid in commercial laying birds in Udi, South Eastern Nigeria. *Comparative Clinical Pathology*, 18, 395–398.

Freitas Neto, O.C., Arroyave, W., Alessi, A.C., Fagliari, J.J. & Berchieri, A., Jr. (2007). Infection of commercial laying hens with *Salmonella* Gallinarum: clinical, anatomopathological and haematological studies. *Poultry Science*, 9, 133–141.

Gast, R.K. (2013). Paratyphoid infections. In D.E. Swayne, J.R. Glisson, L.R. McDougald, L.K. Nolan, D.L. Suarez & V.L. Nair (Eds.). *Diseases of Poultry* 13th edn (pp. 694–713). Ames: Wiley-Blackwell and Iowa State Press.

Gordon, R.F. & Tucker, J.F. (1957). The treatment of chronic carriers of *Salmonella pullorum* with furazolidone. *The British Veterinary Journal*, 113, 99–111.

Greenwood, P.E. & Nikulin, M.S. (1996). *A Guide to Chi-squared Testing* (p. 280). New York, NY: John Wiley & Sons.

Hall, W.J., Legenhausen, D.H. & Macdonald, A.D. (1949). Studies on fowl typhoid I. Nature and dissemination. *Poultry Science*, 28, 344–362.

Hu, J., Bumstead, N., Barrow, P.A., Sebastiani, G., Olien, L., Morgan, K. & Malo, D. (1997). Resistance to Salmonellosis in the chicken is linked to NRAMP1 and TNC. *Genome Research*, 7, 693–704.

Kang, M.S., Kim, A., Jung, B.Y., Her, M., Jeong, W., Cho, Y.M., Oh, J.Y., Lee, Y.J., Kwon, J.H. & Kwon, Y.K. (2010). Characterization of antimicrobial resistance of recent *Salmonella enterica* serovar Gallinarum isolates from chickens in South Korea. *Avian Pathology*, 39, 201–205.

Kim, J.H. & Kim, K.S. (2010). Hatchery hygiene evaluation by microbiological examination of hatchery samples. *Poultry Science*, 89, 1389–1398.

Klein, E. (1889). Über eine epidemische Krankheit der Hühner, verursacht durch einen Bacillus, *Bacillus gallinarum*. *Zentralblatt für Bakteriologie*, 5, 689–693.

Kwon, Y.K., Kim, A., Kang, M.S., Her, M., Jung, B.Y., Lee, K.M., Jeong, W., An, B.K. & Kwon, J.H. (2010). Prevalence and characterization of *Salmonella* Gallinarum in the chicken in Korea during 2000 to 2008. *Poultry Science*, 89, 236–242.

Lee, Y.J., Kim, K.S., Kim, J.H. & Tak, R.B. (2004). *Salmonella* Gallinarum *gyrA* mutations associated with fluoroquinolone resistance. *Avian Pathology*, 33, 251–257.

Lee, Y.J., Kim, K.S., Kwon, Y.K. & Tak, R.B. (2003). Biochemical characteristics and antimicrobials susceptibility of *Salmonella* Gallinarum isolated in Korea. *Journal of Veterinary Science*, 4, 161–166.

Mariani, P., Barrow, P., Cheng, H.H., Groenen, M.A.M., Negrini, R. & Bumstead, N. (1998). A major quantitative trait locus determining resistance to salmonellosis is located on chicken chromosome 5. *Animal Genetics*, 29, 73–74.

Moore, E.N. (1948). The efficacy of recently developed sulfonamides against fowl typhoid. *Poultry Science*, 25, 307–311.

Nonga, H.E., Simon, C., Karimuribo, E.D. & Mdegela, R.H. (2010). Assessment of antimicrobial usage and residues in commercial chicken eggs from smallholder poultry keepers in Morogoro municipality, Tanzania. *Zoonoses and Public Health*, 57, 339–344.

OIE. (2015). *World Organization for Animal Health. Disease Information*. Paris: OIE.

Oliveira, G., Berchieri, A. & Fernandes, A. (2005). Experimental infection of laying hens with *Salmonella enterica* serovar Gallinarum. *Brazilian Journal of Microbiology*, 36, 51–56.

Penha Filho, R.A.C., Ferreira, J.C., Kanashiro, A.M.I., Costa Darini, A.L. & Berchieri, A., Jr. (2016). Antimicrobial susceptibility of *Salmonella* Gallinarum and *Salmonella* Pullorum isolated from ill poultry in Brazil. *Ciência Rural*, 46, 513–518.

Pomeroy, B.S. (1978). Fowl typhoid. In M.S. Hofstad, B.W. Calnek, C.F. Helmbolt, W.M. Reid & H.W. Yoder Jr. (Eds.). *Diseases of Poultry* 7th edn (pp. 100–116). Ames: Iowa State University Press.

Ribot, E.M., Fair, M.A., Gautom, R., Cameron, D.N., Hunter, S.B., Swaminathan, B. & Barret, T.J. (2006). Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathogens and Disease*, 3, 59–67.

Seo, Y.S., Lee, S.H., Shin, E.K., Kim, S.J., Jung, R. & Hahn, T.W. (2006). Pulsed-field gel electrophoresis of *Salmonella* Gallinarum and comparison with random amplified polymorphic DNA. *Veterinary Microbiology*, 115, 349–357.

Shivaprasad, H.L. (2000). Fowl typhoid and pullorum disease. *Revue Scientifique et Technique de l'OIE*, 19, 405–424.

Shivaprasad, H.L. & Barrow, P.A. (2013). Pullorum disease and fowl typhoid. In D.E. Swayne, J.R. Glisson, L.R. McDougald, L.K. Nolan, D.L. Suarez & V.L. Nair (Eds.). *Diseases of Poultry* 13th edn (pp. 678–693). Ames: Wiley-Blackwell and Iowa State Press.

Smith, H.W., Tucker, J.F. & Lovell, M.A. (1981). Furazolidone resistance in *Salmonella* Gallinarum: the relationship between in vitro and in vivo determinations of resistance. *Journal of Hygiene*, 87, 71–79.

Stuart, E.E. & Keenum, R.D. (1970). Preincubation treatment of chicken hatching eggs infected with *Salmonella* Pullorum. *Avian Diseases*, 14, 87–95.

Van Immerseel, F., Studholme, D.J., Eeckhaut, V., Heyndrickx, M., Dewulf, J., Dewaele, I., Van Hoorebeke, S., Haesebrouck, F., Van Meirhaeghe, H., Ducatelle, R., Paszkiewicz, K. & Titball, R.W. (2013). *Salmonella* Gallinarum field isolates from laying hens are related to the vaccine strain SG9R. *Vaccine*, 31, 4940–4945.

Van Roekel, H., Bullis, K.L., Snoeynbos, G.H., Clarke, M.K. & Flint, O.S. (1951). Significant observations in pullorum disease eradication. *Veterinary Medicine*, 46, 18–19.

Wigley, P., Hulme, S., Rothwell, L., Bumstead, N., Kaiser, P. & Barrow, P.A. (2006). Macrophages isolated from chickens genetically resistant or susceptible to systemic salmonellosis show magnitudinal and temporal differential expression of cytokines and chemokines following *Salmonella enterica* challenge. *Infection and Immunity*, 74, 1425–1430.

Wigley, P., Hulme, S.D., Bunstead, N. & Barrow, P.A. (2002). In vivo and in vitro studies of genetic resistance to systemic salmonellosis in the chicken encoded by the SAL1 locus. *Microbes and Infection*, 4, 1111–1120.

Wilson, J.E. (1956). The treatment of carriers of *Salmonella* pullorum and *Salmonella* Gallinarum with furazolidone. *The Veterinary Record*, 68, 748–751.