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A comparative study on the pathogenesis of egg contamination by different serotypes of *Salmonella*

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Salmonella enterica serotype Enteritidis is the predominant serotype associated with egg-borne salmonellosis in humans. Apparently this serotype possesses particular characteristics that increase its chance to contaminate eggs. To identify these characteristics, two *Salmonella* serotype Enteritidis strains as well as one strain of each of the serotypes *Salmonella* Typhimurium, *Salmonella* Heidelberg, *Salmonella* Virchow and *Salmonella* Hadar strain were used to examine different aspects related to egg contamination. After an intravenous infection of laying hens, it was observed that the ability of the serotype Enteritidis strains to colonize the reproductive organs was significantly higher compared with the *Salmonella* Heidelberg, *Salmonella* Virchow and *Salmonella* Hadar strains but not with the *Salmonella* Typhimurium strain. Inoculating low numbers of the different *Salmonella* serotypes in egg albumen at 42°C demonstrated that the growth of the strains belonging to the *Salmonella* serotypes Virchow and Hadar was seriously repressed. The other serotypes, however, survived in albumen for 24 h. Furthermore, using two different specifically designed egg infection models, it was shown that all strains used in this study were able to penetrate into and multiply inside the yolk at 25°C. These findings indicate that the ability to grow in eggs post lay is not specific for the serotype Enteritidis. In conclusion, comparing strains belonging to different *Salmonella* serotypes has revealed that most probably a preferential colonization of the reproductive organs and an enhanced survival at 42°C allows the serotype Enteritidis to contaminate eggs.

Introduction

Over the past two decades, *Salmonella enterica* serotype Enteritidis became an important cause of food-borne human illness worldwide, mostly traced back to egg consumption (Angulo & Swerdlow, 1999). Most reported *Salmonella* outbreaks in humans in the past two decades were caused by *Salmonella* Enteritidis, while outbreaks due to other *Salmonella* serotypes were rather low (Rodrigue *et al.*, 1990; Henzler *et al.*, 1994). In response to this pandemic, the European Union introduced Regulation (EC) No. 2160/2003 to ensure that proper and effective measures were taken at all stages of the poultry production chain, in order to reduce the prevalence and the risk that *Salmonella* poses to public health. Following this regulation, member states set up national programmes. As a result, in the last years a considerable reduction in the number of *Salmonella* Enteritidis isolates from humans was reported in some countries. As an example, in Belgium, during 2005, a 71% decrease in human *Salmonella* Enteritidis infections compared with the average annual number of cases in the period 2000 to 2004 was recorded (Collard *et al.*, 2008).

In spite of considerable research efforts, the actual mechanism of the *Salmonella* Enteritidis egg tropism is still unclear. Two possible infection routes have been considered: penetration through the eggshell from the colonized cloaca or from faeces, during or after oviposition; or direct contamination of yolk, albumen, eggshell membranes or eggshells during egg formation originating from infected reproductive organs. Numerous studies on natural and experimental infection with *Salmonella* Enteritidis have provided fragmentary information in support of one or the other. Although many studies have been performed showing the ability of *Salmonella* Enteritidis to penetrate eggshells (De Reu *et al.*, 2006), colonize the reproductive tract and contaminate eggs (Keller *et al.*, 1995; Miyamoto *et al.*, 1997; Okamura *et al.*, 2001a,b), survive in albumen (Kang *et al.*, 2006) and migrate from albumen to yolk to multiply in eggs after storage (Gast *et al.*, 2005), it is striking that few studies have actually investigated whether any of the phenomena is specific for *Salmonella* Enteritidis and thus gives *Salmonella* Enteritidis an advantage over other serotypes in terms of capability of egg contamination.

In the present study, a range of different bioassays, both *in vivo* and *in vitro*, was used to study different aspects of the pathogenesis of egg contamination comparing strains belonging to five different *Salmonella* serotypes: *Salmonella* Enteritidis (two strains), *Salmonella* Typhimurium, *Salmonella* Heidelberg, *Salmonella* Virchow and *Salmonella* Hadar. The tests included reproductive organ colonization after infection *in vivo*,

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invasion and proliferation in primary tubular magnum and isthmus cells, survival in egg albumen at 42°C, and migration, penetration and multiplication in eggs post lay.

Materials and Methods

Bacterial strains. Six isolates from five different *Salmonella enterica* serotypes were used in this study. All strains are originally isolated from poultry. Two strains belonged to the serotype Enteritidis phage type 4: strain 147, originally isolated in Germany from egg content (Methner *et al*., 1995), and S1400 from the UK, a poultry field strain (Allen-Vercoe & Woodward, 1999). The four other strains belonged to the serotypes Typhimurium (strain SL1344; Hoiseth & Stocker, 1981), Heidelberg (strain 704Sa06 isolated from broiler faeces), Virchow (strain 2018Sa06 isolated from egg contents) and Hadar (strain 26Sa06 isolated from faeces in a parent flock). The latter three strains were kindly provided by Dr H. Imberechts from the Belgian Reference Laboratory for *Salmonella*, Animal Health (Veterinary and Agrochemical Research Centre, Ukkel, Belgium).

Intravenous infection trial. *Animals.* One hundred and forty-four ISA Brown laying hens that were not vaccinated against *Salmonella* were housed on a litter floor from the age of 18 weeks until the end of the experiment. At the day of arrival, the *Salmonella* status of all the animals was tested by bacteriological analysis of cloacal swabs. Sera of 20% of the animals were tested for anti-*Salmonella* antibodies using a previously described enzyme-linked immunosorbent assay (Desmidt *et al*., 1996). The animals had free access to drinking water and were fed *ad libitum*. They received a 16 h light/8 h dark lighting scheme.

Experimental design. The laying hens were randomly divided into six groups of 24 chickens and were housed in separate rooms. At the age of 22 weeks, each group of birds was intravenously inoculated with 1 ml of 1×10^8 colony-forming units (CFU)/ml of one of the above described *Salmonella* strains. At days 4, 7 and 14 post infection (p.i.), five to eight birds per group were euthanized by intravenous embutramid injection (T61; Intervet, Belgium). Samples of the spleen, oviduct and ovary were taken for bacteriological analysis. Throughout the study period, eggs were collected daily and analysed for the presence of *Salmonella*.

Bacteriological analysis of organs and eggs. Samples of the spleen, oviduct and ovary were weighted. The spleen and ovary were homogenized with a stomacher in 10 times the volume of buffered peptone water (BPW) (Oxoid, Basingstoke, UK). The oviduct samples were sliced into very small pieces and subsequently homogenized in a stomacher. The number of CFU *Salmonella*/g tissue was determined by plating 10-fold serial dilutions on brilliant green agar (BGA) (Lab M Limited, Bury, UK). Samples that tested negative after direct plating were pre-enriched overnight at 37°C in BPW and afterwards were enriched in tetrathionate brilliant green broth (1/10) (Merck, Darmstadt, Germany) by overnight incubation at 37°C. Then, a loopful of the tetrathionate brilliant green broth was streaked onto BGA.

Upon collection, faeces on the surface of the eggs were removed with a scalpel and the shell was decontaminated with 70% ethanol. Then the eggs were broken aseptically and the content of one egg was pooled in a sterile plastic bag. The contents were homogenized with a stomacher during 3 min. Forty millilitres of BPW was added to the egg content of each egg and incubated for 48 h at 37°C. Further enrichment in tetrathionate brilliant green broth was performed overnight at 37°C. To detect the *Salmonella* bacteria, a loopful of the broth was plated on BGA. Agglutination was used to confirm the serotype.

Invasion in tubular gland cells of the isthmus and magnum. *Magnum and isthmus tubular gland cell isolation.* Magnum and isthmus tubular gland cells were obtained using the method of Jung-Testas *et al.* (1986). Seven-day-old chicks were given daily one subcutaneous injection of 1 mg oestradiol benzoate (Sigma, St Louis, Missouri, USA) in 0.5 ml sesame oil during 10 days. After 3 weeks, the chickens received a second stimulation by reinjection with the same daily amount of oestradiol benzoate for seven consecutive days. Hereafter the chicks were euthanized by intravenous injection of T61. The oviducts were aseptically removed and the magnum and isthmus were separated. The segments were slit open longitudinally and washed three times in Hank's buffered salt solution (HBSS) (Gibco, Invitrogen, Auckland, New Zealand). Afterwards, both segments of the oviduct were cut into very small pieces. Subsequently, the tissue was dissociated for 1 h at 37 °C with 1 mg/ml (180 u/mg solid) collagenase (Sigma), diluted in minimal essential medium (DMEM) (Gibco, Invitrogen) with penicillin–streptomycin (50μg/ml) (Gibco, Invitrogen). The tissue was allowed to sediment and the supernatant was removed and replaced with HBSS containing trypsin (0.25%) (Gibco, Invitrogen) and ethylenediamine tetraacetic acid (3mM). Again the tissue was dissociated by shaking the cells during 20 min at 37°C. To neutralize the trypsin, 2 ml foetal calf serum (FCS) (Gibco, Invitrogen) was added after complete dissociation. To remove the red blood cells, the tissue was treated with lysis buffer (0.87% NH_4Cl and 0.1% $NaHCO_3$). Finally, the resulting cell suspension was filtered using a cell strainer (Ø70 μm) (BD Falcon, San Jose, California, USA) and centrifuged at $3000 \times g$. The cell pellets were rinsed twice with DMEM containing 10% FCS. The cells were seeded at 1×10^6 cells/ml in tissue culture flasks in DMEM supplemented with 15% FCS, insulin (0.12 IU/ml) (Sigma), and oestradiol (50 nM) (Sigma). This is subsequently called complete medium. Penicillin–streptomycin and fungizone (amphotericin B, 250 ng/ml) (Gibco, Invitrogen) were added to the medium to avoid contamination of the primary cells. The plates were placed in the cell incubator at 37°C, 5% CO_2. After 2 h, fibroblasts were attached to the cell culture flask. The non-adhering oviduct cells were removed and seeded into tissue culture 96-well plates.

Invasion assays. At day 2 post isolation, the cells were rinsed three times with DMEM and complete medium without antibiotics and fungizone was added to the cells. At day 3 post isolation, the wells were checked for confluent growth. The different *Salmonella* isolates were grown for 20 h at 37°C in LB, after which the bacterial suspensions were diluted 1:50 in Luria Bertani (LB) (Sigma) and grown for 4 h on a shaker platform ($300 \times g$). The suspensions were centrifuged and diluted in DMEM. The number of CFU/ml was determined by plating 6×20 μl of a dilution series of the suspensions on BGA, after which the plates were incubated overnight at 37°C. The suspensions were kept at 4°C until the next day. The bacterial suspensions were diluted to a density of 5×10^6 CFU/ml in complete medium without FCS and used to infect the magnum and isthmus tubular gland cells. Contact with the cell layer was obtained by 10 min centrifugation at 37°C. Hereafter the plates were incubated for 1 h at 37°C. Then the cells were rinsed three times with DMEM. The remaining extracellular bacteria were killed by replacement of the medium with complete medium containing 100 μg/ml gentamicin (Sigma). After 30 min incubation at 37°C, 5% CO_2, the gentamicin concentration was reduced to 16 μg/ml gentamicin and the cells were subsequently incubated for another 30 min. Subsequently, the cells were rinsed five times with HBSS and lysed with 100 μl of 1% Triton X-100 (Sigma) in distilled water. During 10 min, the plates were shaken at maximum speed on an MTS 2/4 digital microtitre plate shaker. Afterwards, 100 μl HBSS was added to the wells. From this lysate, 10-fold dilution series were made. From each dilution, 6×20 μl was inoculated on BGA, to determine the number of CFU *Salmonella*/ml. The reported values are the means of two independent experiments with three repeats per experiment.

Survival of bacteria in albumen at 42°C. The six *Salmonella* strains were incubated at 42°C in egg albumen and tested on their survival during 24 h. This was done to simulate the conditions in the oviduct during the egg formation. Freshly laid eggs were obtained from a local farm and were surface disinfected by immersion in 70% ethanol, dried and then cracked into a sterile container. Egg albumen from four eggs was pooled and mixed during 3 min using a vortex. The six different *Salmonella* isolates were grown overnight in LB, 37°C at $300 \times g$, and resuspended in phosphate-buffered saline (PBS) to ensure that all medium was removed before inoculation in egg albumen. The bacterial suspensions were mixed with 2 ml egg albumen to a concentration of approximately 1×10^2 and 1×10^3 CFU/ml, respectively, because at higher concentrations the albumen loses its bactericidal activity (Kang *et al.*, 2006). The bacteria–egg albumen mixtures were incubated at 42°C, and at 24 h

samples of the mixtures were taken to determine the concentration of the surviving bacteria by plating serial dilutions on BGA plates. The reported values are the means of three independent experiments.

Penetration into the egg yolk. This *in vitro* egg contamination model was described by Gast *et al.* (2005). Freshly collected eggs obtained from a local farm were aseptically broken and the yolk and albumen were separated. The yolk was carefully transferred into a sterile 50 ml Falcon tube. The vitelline membrane of each yolk was then inoculated with 100 µl PBS containing approximately 100 cfu/ml *Salmonella* bacteria. After the inoculation, yolk samples were kept for 5 min at room temperature. Then, the albumen from a single egg was gently poured into the Falcon tube. Thirty egg-content samples were inoculated with each of the six *Salmonella* strains. After 24 h, 48 h and 96 h incubation at 25°C, 10 yolk samples per strain and per time point were tested for *Salmonella*. Each incubated egg sample was poured into a sterile plastic Petri dish. A small area of the yolk membrane was sealed with a flame-heated spatula to kill the surface bacteria in that region. A sterile syringe was then inserted into the sterile hole of the membrane to remove 2 ml interior yolk contents. The concentration of bacteria was determined by plating 10-fold serial dilutions on BGA plates. Samples that were negative after direct plating, were pre-enriched in 2 ml BPW and were incubated for 2 days at 37°C. Afterwards, these samples were further enriched in tetrathionate brilliant green broth and incubated overnight at 37°C. A loopful of this suspension was then plated on BGA plates. This assay was repeated three times.

Growth of bacteria in whole eggs at room temperature. A previously described *Salmonella* egg growth assay was used (Cogan *et al*., 2001). Bacterial cultures of each *Salmonella* strain were incubated overnight at 37°C on a shaker platform (300 × *g*) and serial dilutions in PBS were prepared to give suspensions of 15 CFU/ml. Two hundred microlitres, equalling about three cells per egg, of the *Salmonella* suspensions were inoculated into the albumen of eggs using a 1 ml syringe and 26 G × 0.5″ needle. The size of the inoculum was verified for each strain by plating 10 × 200 µl onto BGA plates. Eggs, obtained from a local farm, were inspected on arrival at the laboratory and cracked or dirty eggs were discarded. Shells were surface-sterilized with 70% ethanol that was allowed to evaporate. A small hole was punched in the pointed end of the egg with a sterile pin and 200 µl bacterial suspension was introduced into the albumen. The hole was then sealed with warm paraffin. Eggs were subsequently stored at a constant temperature of 25°C for 8 days. Eggs were surface sterilized with ethanol, aseptically cracked and placed in a sterile plastic bag. Then the contents were homogenized during 3 min using a stomacher. Ten-fold dilutions of the homogenate were made in PBS and 6 × 20 µl was plated on BGA plates. Egg samples that were negative after direct plating were enriched as described above. In accordance with previous work by Board (1964), concentration levels higher than 10^6 *Salmonella* bacteria/ml whole egg contents were used to indicate growth occurring as a result of *Salmonella* accessing the yolk contents. Eggs that showed no growth were excluded from the statistical analysis as it was assumed that these eggs received no inoculum. Twenty eggs were inoculated with each bacterial strain in each experiment. Experiments were performed three times.

Statistical analysis. The data from the *in vivo* infection trial were analysed with SPSS 12.0 software using a one-way analysis of variance (ANOVA) test to compare the means of the $\log_{10}$ values of *Salmonella* in the different organs, between the different groups. Binary regression was used to compare the numbers of positive eggs that were laid after challenge in the different serotype groups. Serotype differences of the invasion assay and the survival assay in albumen were also analysed with the one-way ANOVA test. To analyse the data of the penetration assay and the egg growth assay, Statistix 8.2 software was used. The two-sample proportion test was used to compare the differences in the number of eggs in where yolk invasion occurred. Significant differences between the mean concentrations of the *Salmonella* strains in the interior yolk contents after 96 h were determined by applying the one-way ANOVA test.

Results

***In vivo* intravenous infection trial.** *Mortality, egg production and percentage positive eggs.* Over the whole experiment, the Typhimurium group showed the highest mortality. Seven birds died after the infection (29%), three at day 1 p.i. and four at day 2 p.i. Furthermore, five animals inoculated with *Salmonella* Enteritidis S1400 (20%) and two animals infected with *Salmonella* Enteritidis 147 (8%) died within 2 days of the infection. In the groups infected with *Salmonella* Heidelberg and *Salmonella* Virchow, one hen per group died on day 1 and day 3 p.i., respectively, and no birds died in the *Salmonella* Hadar group. The egg production was dramatically decreased in all groups after infection and did not recover again within 2 weeks after infection. Throughout the whole experiment, four of five eggs in the *Salmonella* Enteritidis 147 group, three of five eggs in the *Salmonella* Enteritidis S1400 group, two of five eggs in the *Salmonella* Typhimurium group, zero of six eggs in the *Salmonella* Heidelberg group, one of 16 eggs in the *Salmonella* Virchow group and zero of 16 eggs in the *Salmonella* Hadar group were positive for the respective serotype.

Colonization of the spleen and reproductive organs. There was no significant difference in spleen colonization between the groups inoculated with the six *Salmonella* strains for all time points. The $\log_{10}$ values of the numbers of CFU *Salmonella*/g spleen are presented in Table 1. The different serotypes colonized the reproductive organs at a different level. Mean bacterial counts in the reproductive tissues are presented in Table 1. Four days after infection, a difference was observed between the Enteritidis strains and the Heidelberg, Virchow and Hadar serotypes but it was not significant, probably due to the low number of animals at that time point. One week after the infection, both *Salmonella* Enteritidis strains were colonizing the oviduct significantly ($P < 0.05$) more than the serotypes Heidelberg, Virchow and Hadar. There was no significant difference between oviduct colonization of the Enteritidis serotype strains and the Typhimurium serotype strain. At 14 days p.i., only the *Salmonella* Enteritidis S1400 showed a significantly ($P < 0.05$) higher colonization level of the oviduct compared with the serotype Typhimurium, Heidelberg, Virchow and Hadar strains. Significant differences ($P < 0.05$) in the ovary samples were also observed between several serotype groups. Both strains of the serotype Enteritidis and the strain of the serotype Typhimurium were colonizing the ovaries significantly more than the Hadar serotype at day 4 p.i., and the Heidelberg serotype at day 7 p.i. Figure 1 represents the ratios of oviduct versus spleen and ovary versus spleen colonization at 4, 7 and 14 days after intravenous inoculation. In these figures, value 1 means 10-fold more bacteria in the oviduct/ovary compared with the spleen, and value 2 means 100-fold, while negative values mean that more bacteria are colonizing the spleen compared with the oviduct/ovary.

Invasion in magnum and isthmus tubular gland cells. *Salmonella* bacteria were isolated from the isthmus and magnum glandular cells, and the number of intracellular bacteria was compared between the five serotypes. There was no significant difference in the number of invaded

Table 1. *Mean log_{10} CFU/g* Salmonella *serotypes in the spleen, oviduct and ovary samples at days 4, 7 and 14 after intravenous inoculation*

	Day 4			Day 7			Day 14		
Serotype and strain	Spleen	Oviduct	Ovaria	Spleen	Oviduct	Ovaria	Spleen	Oviduct	Ovaria
Salmonella Enteritidis 147	4.83 ± 0.52^{A}	4.42 ± 1.38^{A}	5.11 ± 1.98^{A}	4.19 ± 0.53^{A}	4.64 ± 0.79^{A}	5.02 ± 1.42^{A}	3.78 ± 0.30^{A}	2.99 ± 2.99^{AB}	5.35 ± 0.79^{A}
Salmonella Enteritidis S1400	5.00 ± 0.73^{A}	4.86 ± 0.95^{A}	4.59 ± 1.38^{A}	3.64 ± 0.42^{A}	4.45 ± 0.57^{A}	6.46 ± 1.07^{A}	2.92 ± 0.76^{A}	4.96 ± 1.53^{B}	4.51 ± 2.19^{A}
Salmonella Typhimurium	5.07 ± 0.43^{A}	4.47 ± 1.83^{A}	4.91 ± 1.63^{A}	3.81 ± 0.48^{A}	3.48 ± 1.30^{AB}	5.84 ± 1.66^{A}	2.57 ± 0.59^{A}	2.10 ± 1.27^{A}	4.86 ± 2.33^{A}
Salmonella Heidelberg	4.81 ± 0.34^{A}	2.53 ± 0.86^{A}	2.74 ± 1.01^{AB}	4.24 ± 0.31^{A}	1.52 ± 1.41^{B}	2.14 ± 1.35^{B}	3.58 ± 0.79^{A}	2.11 ± 1.06^{A}	4.48 ± 2.24^{A}
Salmonella Virchow	4.88 ± 0.72^{A}	2.48 ± 1.83^{A}	3.54 ± 1.51^{AB}	3.74 ± 0.57^{A}	2.09 ± 1.81^{B}	4.79 ± 2.39^{AB}	3.09 ± 0.64^{A}	1.66 ± 1.52^{A}	3.01 ± 1.56^{A}
Salmonella Hadar	4.81 ± 0.41^{A}	2.61 ± 1.92^{A}	1.98 ± 0.91^{B}	4.13 ± 0.32^{A}	1.81 ± 1.63^{B}	3.62 ± 1.63^{AB}	3.25 ± 0.24^{A}	1.68 ± 1.45^{A}	3.04 ± 2.32^{A}

Data presented as the mean log value of the numbers of isolated bacteria/g tissue ± standard deviation. Values with different superscript uppercase letters are statistically different from each other within the same column ($P < 0.05$).

bacteria between the serotypes, indicating that all *Salmonella* serotypes invaded the tubular gland cells to the same extent. The mean log values of the number of invaded *Salmonella* bacteria varied between 5.0 and 5.6 for the magnum and 5.1 and 5.8 for the isthmus.

Survival of bacteria in albumen at 42°C. The six *Salmonella* strains were incubated at 42°C in egg albumen and were tested for their survival after 24 h (Figure 2a,b). Similar results were obtained with 10^2 CFU/ml and 10^3 CFU/ml as the bacterial concentrations of the

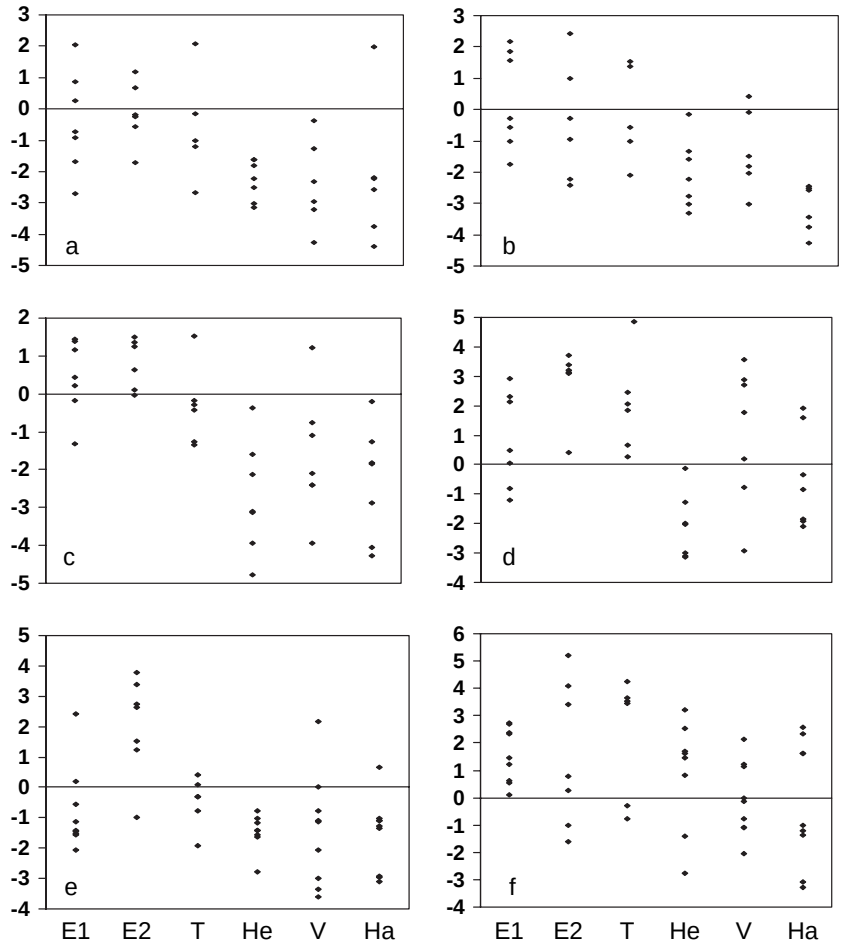

Figure 1. *Representation of the preferential colonization of the reproductive organs by the* Salmonella enterica *strains. 1a, 1c and 1e: each dot represents the difference between log_{10} CFU/g oviduct and log_{10} CFU/g spleen of one laying hen. 1b, 1d and 1f: each dot represents the difference between log_{10} CFU/g ovaria and log_{10} CFU/g spleen of one laying hen. 1a and 1b: day 4 p.i., 1c and 1d: day 7 p.i., and 1e and 1f: day 14 p.i. after intravenous inoculation with 1×10^8 CFU of one of the* Salmonella *isolates:* Salmonella *Enteritidis 147 (E1),* Salmonella *Enteritidis S1400 (E2),* Salmonella *Typhimurium (T),* Salmonella *Heidelberg (He),* Salmonella *Virchow (V) and* Salmonella *Hadar (Ha).*

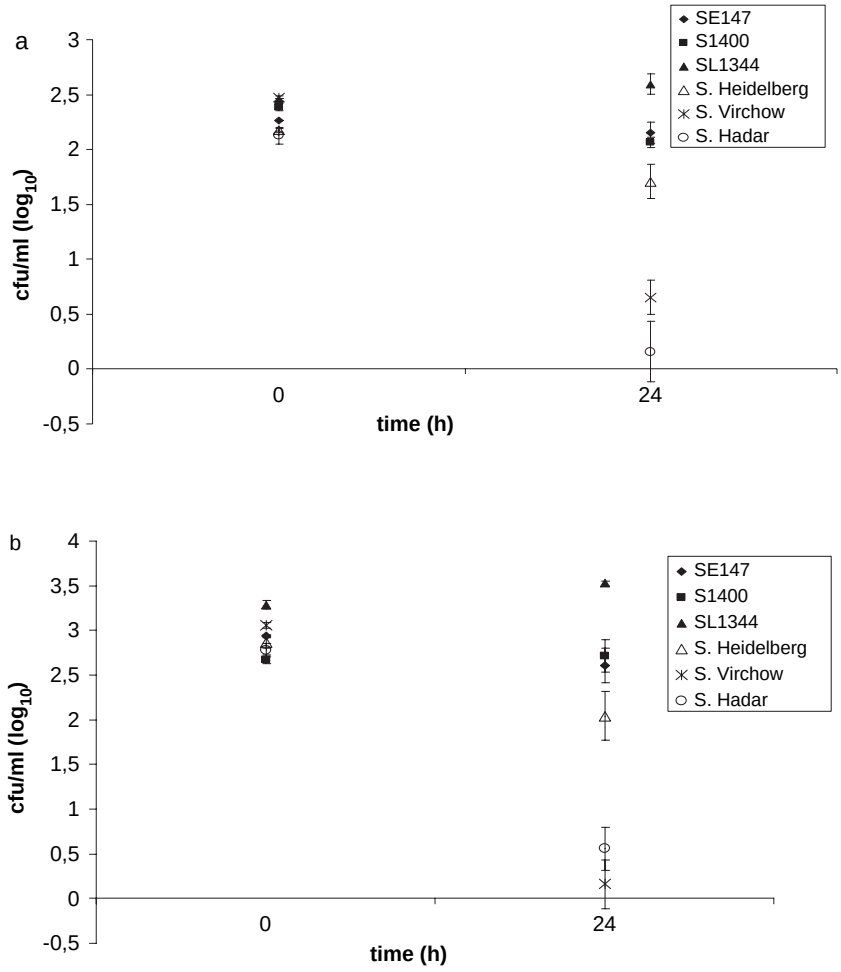

Figure 2. *Mean log_{10} CFU/ml of the different* Salmonella *serotypes at time 0 h and 24 h after inoculation of (2a) 10^2 CFU/ml and (2b) 10^3 CFU in egg albumen at 42°C. Error bars present the standard error of the means.*

inoculum. The two *Salmonella* Enteritidis strains displayed similar survival but showed significantly higher survival than the serotypes Virchow and Hadar. After 24 h, there was a 100-fold to 1000-fold difference between the *Salmonella* Enteritidis strains and the *Salmonella* strains belonging to the serotypes Virchow and Hadar. This means that the serotypes Virchow and Hadar strains are killed much faster in the egg albumen than the serotype Enteritidis strains. On the contrary, after 24 h, the bacterial counts of the *Salmonella* Enteritidis serotype strains in egg white were significantly lower than the numbers of the *Salmonella* Typhimurium strain. Furthermore, we observed no significant difference between the survival capacities of the *Salmonella* Enteritidis strains and the *Salmonella* Heidelberg strain.

Penetration into the egg yolk. After 24 h and 48 h of incubation at 25°C, none of the six strains were recovered from the yolk contents in any of the whole egg samples. After 96 h, however, all the strains were found in the yolk (Table 2). The only significant difference observed was between *Salmonella* Enteritidis 147 and the *Salmonella* Virchow strain. Their recovery rates after 96 h were 65% and 30%, respectively. Furthermore, the mean log_{10} concentrations of both Enteritidis serotypes found in the yolk contents were significantly higher than the bacterial concentration of the strain of the serotype Virchow.

Growth of bacteria in whole eggs at room temperature. Sixty eggs were inoculated and the number of *Salmonella* bacteria enumerated. The frequencies of yolk invasion are summarized in Table 3. Except for the serotype Typhimurium strain, no significant differences in yolk invasion between the other serotypes were observed. Only one egg tested positive for *Salmonella* Typhimurium after 8 days of storage at room temperature (1.7%). This was a significantly lower percentage than that of the *Salmonella* Enteritidis 147 and S1400, *Salmonella* Heidelberg and *Salmonella* Hadar isolates, which invaded

Table 2. *Isolation and enumeration of the* Salmonella *bacteria in the yolk contents after inoculation with approximately 10^2 CFU onto the vitelline membrane and incubation at 25°C during 96 h*

Serotype and strain	*Salmonella*-positive yolk samples/total	*Salmonella* concentration in yolk samples (log_{10} CFU/ml)
Salmonella Enteritidis 147	$13/20^{A}$	2.04^{B}
Salmonella Enteritidis S1400	$10/20^{AB}$	1.88^{B}
Salmonella Typhimurium	$9/20^{AB}$	1.59^{AB}
Salmonella Heidelberg	$10/20^{AB}$	1.60^{AB}
Salmonella Virchow	$6/20^{B}$	0.73^{A}
Salmonella Hadar	$10/20^{AB}$	1.38^{AB}

Values with different superscript uppercase letters are statistically significant different from each other within the same column ($P < 0.05$).

Table 3. *Number of eggs showing growth of different* Salmonella *serotypes to high numbers ($>10^6$ CFU/ml), indicative of invasion of the egg yolk by* Salmonella

Strain	*Salmonella*-positive egg content samples ($>10^6$ CFU/ml)/total
Salmonella Enteritidis 147	$9/60^{A}$
Salmonella Enteritidis S1400	$8/60^{A}$
Salmonella Typhimurium	$1/60^{B}$
Salmonella Heidelberg	$10/60^{A}$
Salmonella Virchow	$5/60^{AB}$
Salmonella Hadar	$8/60^{A}$

For each bacterial isolate, 60 eggs were inoculated with approximately three *Salmonella* cells, and stored for 8 days at 25°C. Values with different superscript uppercase letters are statistically significant different from each other ($P<0.05$).

and multiplied in the yolk between 13.3% and 16.7%. The second lowest frequency of yolk invasion was displayed by the serotype Virchow (8.3%).

Discussion

The present study was designed to provide insights into the specific mechanisms that allow serotype Enteritidis strains to contaminate eggs more successfully than strains belonging to any other serotype. This was done by comparing two strains of *Salmonella* Enteritidis with four strains of other serotypes (*Salmonella* Typhimurium, Heidelberg, Virchow and Hadar). Several infection models, both *in vivo* and *in vitro*, were used to investigate different steps in the pathogenesis of egg contamination. It has been clear that eggshell and membrane penetration are not a unique property of *Salmonella* Enteritidis, and that other *Salmonella* serotypes and totally unrelated bacteria can traverse these barriers in the used models (De Reu *et al*., 2006). Therefore, in this study, we have only focused on the different aspects of egg contamination through vertical transmission.

In a first experiment, the colonization of the reproductive tract was investigated after an intravenous challenge with the different serotypes. Intravenous infection was used since high levels of oviduct infection were obtained until several weeks after inoculation and, most probably, oral infection would not result in high enough bacterial numbers in the reproductive tract. In addition, intravenous infection results in significant contamination of eggs, in contrast to oral infection (De Buck *et al.*, 2004). In our study, the spleen was colonized to the same extent by all serotypes after intravenous infection, suggesting that the ability to colonize internal organs is not specific for the serotype Enteritidis strains. In contrast, both *Salmonella* Enteritidis isolates showed a higher colonization of the reproductive organs (oviduct and ovary) in comparison with the *Salmonella* Heidelberg, Virchow and Hadar serotype isolates. In addition, the Enteritidis strains presented specific tropism for the reproductive organs as they are recovered in higher numbers from oviduct and ovary than from spleen. This was not the case for the *Salmonella* Heidelberg, Virchow and Hadar serotype strains. These observations are in accordance with a previous study by Okamura *et al.* (2001a,b) demonstrating that, among six serotypes, *Salmonella* Enteritidis was the predominant serotype to colonize the reproductive organs after an intravenous and intravaginal infection. It is in contrast with other studies reporting that the serotype Enteritidis colonized the reproductive tissues equally well as the serotype Heidelberg (Gast *et al.*, 2004). The *Salmonella* Heidelberg strains, however, were recovered from contaminated eggs at a lower frequency than the *Salmonella* Enteritidis strain. We cannot, however, compare these results since the birds were inoculated orally with different strains than the ones used in our study. Therefore, the conflicting results seen in the different experimental studies are possibly due to differences in strains used, inoculation route or inoculation dose. No significant difference was observed in oviduct colonization between the Enteritidis serotype strains and the Typhimurium serotype strain, except at day 14 p.i., at which point only one *Salmonella* Enteritidis strain showed a significantly higher colonization level of the oviduct compared with the *Salmonella* Typhimurium strain. This finding is in agreement with a study by Keller *et al.* (1997) demonstrating that *Salmonella* Enteritidis and *Salmonella* Typhimurium were equal in their potential to colonize the tissues of the reproductive tract and to infect forming eggs in the oviduct prior to oviposition. *Salmonella* Enteritidis, however, but not *Salmonella* Typhimurium, was isolated from laid eggs. The results of this previous study and our study indicate that *Salmonella* Typhimurium can equally colonize the reproductive tract tissues as *Salmonella* Enteritidis, but the underlying reason why serotype Enteritidis and not serotype Typhimurium is associated with eggs remains unclear.

The interaction between *Salmonella* Enteritidis and the oviduct tissue may play an important role in the ability of *Salmonella* Enteritidis to contaminate eggs. Association of the serotype Enteritidis with tubular gland cells of the oviduct has been observed after natural infection (Hoop & Pospischil, 1993) and after experimental infection (Keller *et al.*, 1995; De Buck *et al.*, 2004). Moreover, it has already been shown that *Salmonella* Enteritidis can invade tubular gland cells of the oviduct and that interactions between type I fimbriae and mannose-containing residues of the oviduct secretions are involved in colonization of the oviduct (De Buck *et al.*, 2003, 2004). In the present study, however, using a tubular gland cell primary culture model, we showed that the four *Salmonella* serotypes tested were equally able to invade the magnum and isthmus tubular gland cells as the serotype Enteritidis. In addition, all serotypes tested proliferated inside the epithelial cells (data not shown). These results suggest that invasion and proliferation in oviduct tissue is most probably not a unique characteristic of the serotype Enteritidis. These findings are in contrast with a study by Mizumoto *et al.* (2005) showing that the adhesion and invasion of *Salmonella* Enteritidis in the vaginal epithelium was significantly higher in comparison with several other *Salmonella* serotypes.

Another feature that may be one of the biological determinants contributing to the epidemiological association of *Salmonella* Enteritidis with eggs is survival in egg albumen at 42°C. Keller *et al.* (1997) reported that *Salmonella* Typhimurium contaminated forming eggs (before they were laid) at higher frequencies than *Salmonella* Enteritidis in experimentally infected hens. Nevertheless, only the serotype Enteritidis could be isolated from laid eggs. Therefore, it was hypothesized

that the association of the serotype Enteritidis with chicken eggs may be due to its enhanced survival ability in egg white during the egg formation. In the present study, the bactericidal effect of the albumen at chicken body temperature was examined for five *Salmonella* serotypes. The initial bacterial concentrations used were low since, at higher concentrations, the antimicrobial factors of the egg albumen may saturate and thus result in a reduced killing (Kang *et al.*, 2006). Remarkably, the *Salmonella* Enteritidis strains, the *Salmonella* Typhimurium strain and the *Salmonella* Heidelberg strain could survive in the hostile environment of egg albumen for 24 h. This is the average length of time for natural egg formation in the chicken oviduct. The strains of serotypes Virchow and Hadar were much more susceptible to the antimicrobial activity of egg white and, after 24 h, almost all bacteria were killed. This could explain why in natural infections *Salmonella* Virchow and *Salmonella* Hadar are almost never associated with eggs. Another remarkable result is that the *Salmonella* Typhimurium strain survived better than the *Salmonella* Enteritidis strains. This finding is in agreement with Guan *et al.* (2006), who demonstrated that the survival characteristics in egg white at 37°C and 42°C were similar for several *Salmonella* Enteritidis and *Salmonella* Typhimurium strains. However, this finding is in contrast with a study by Lu *et al.* (2003) demonstrating an enhanced survival of the serotype Enteritidis at 37°C. This enhanced resistance to egg albumen was associated with the presence of YafD, a putative DNA repair enzyme. In addition, Clavijo *et al.* (2006) showed that survival in egg albumen at 37°C was higher for the serotype Enteritidis compared with the serotype Typhimurium and with *Escherichia* coli. These contradictory results probably indicate that strains within a *Salmonella* serotype often differ widely in several aspects of the pathogenesis. Therefore it would be unwise to draw any conclusions on the survival abilities of the serotypes Enteritidis and Typhimurium based on a study using only one or two strains. Nevertheless, our results clearly show that *Salmonella* Virchow and *Salmonella* Hadar are much more susceptible to egg albumen than the other *Salmonella* serotypes tested in this study. The *Salmonella* Heidelberg strain was also able to survive in egg white during 24 h. In the United States, *Salmonella* Heidelberg has recently been identified (Baron *et al.*, 1997) as another significant egg-associated pathogen (Chittick *et al.*, 2006). A possible reason could be its survival in egg white at 42°C.

It is believed that, although some eggs are contaminated within the yolk due to ovarian infection, in the majority of eggs infected via the oviduct tissues, *Salmonella* bacteria are deposited within the albumen (Gast & Holt, 2000). *Salmonella* is unable to multiply in the albumen of fresh eggs and therefore must gain access to the yolk contents in order to multiply to a high level. To compare the ability of different *Salmonella* serotypes to multiply in eggs (i.e. the survival within and the motility from the albumen to the yolk, as well as adherence to and penetration through the vitelline membrane), we have used two different models. Penetration of *Salmonella* Enteritidis through the vitelline membrane has been reported in several previous studies (Braun & Fehlhaber, 1995; Gast *et al.*, 2005). Cogan *et al.* (2004) demonstrated that curli fimbriae are needed to attach to the vitelline membrane, in order to gain access to the yolk. As the genes for curli fimbriae production appear to be ubiquitous within the genus *Salmonella* (Doran *et al.*, 1993), it may be expected that this characteristic is not unique for the serotype Enteritidis. Indeed, this hypothesis was confirmed in our study since all six *Salmonella* isolates were able to penetrate into the interior yolk contents after inoculation onto the exterior surface of the vitelline membrane. In addition, our findings are in agreement with previous research showing that serotypes, other than Enteritidis, are able to penetrate the vitelline membrane and multiply in yolk contents (Gast *et al.*, 2005; Guan *et al.*, 2006; Murase *et al.*, 2006). In a second experiment, the multiplication of the different *Salmonella* serotypes was assessed by inoculating very small numbers of *Salmonella* cells in whole eggs. Flagella are thought to be required for migration towards the vitelline membrane (Cogan *et al.*, 2004), but also these structures are common to most serotypes. Even though in the present study the strain of the serotype Typhimurium multiplied less compared with all other serotypes when inoculated in eggs, no significant difference was observed between the other serotypes, suggesting that the multiplication strategies inside eggs at 25°C are not unique for the serotype Enteritidis.

The purpose of the present studies was to determine what aspects of the pathogenesis of egg contamination enable *Salmonella* Enteritidis strains to infect eggs more efficiently than strains belonging to other *Salmonella* serotypes. In our study, the tested *Salmonella* serotypes Enteritidis and Typhimurium strains colonize the reproductive organs better than the *Salmonella* strains belonging to the serotypes Heidelberg, Virchow and Hadar. In addition, the present study indicates that low numbers of the strains of *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Heidelberg could survive in the egg albumen during egg formation while the *Salmonella* strains belonging to the serotypes Virchow and Hadar were killed much faster. This means that, in natural infections, when the bacterial concentrations in forming eggs are lower, *Salmonella* Virchow strains and *Salmonella* Hadar strains could be killed before oviposition. This could provide us with an explanation why *Salmonella* strains belonging to the serotypes Virchow and Hadar are nearly never isolated from intact eggs. However, the exact mechanism allowing *Salmonella* Enteritidis to contaminate eggs more effectively than the closely related serotype Typhimurium remains to be defined, and large-scale studies using multiple strains from different serotypes should be carried out.

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