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A critical review of *Salmonella* Typhimurium infection in laying hens

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## Abstract

*Salmonella* Typhimurium has been reported to contaminate egg production across the World, but where *Salmonella* Enteritidis is endemic it is this latter serovar that dominates egg-borne salmonellosis. However, *Salmonella* Typhimurium is a major foodborne pathogen so it is important to understand how it can impact the microbiological safety of eggs and what serovar-specific control strategies may be appropriate in the future as control over *Salmonella* Enteritidis continues to improve. To that end, the present review examines the published literature on *Salmonella* Typhimurium in laying hens and eggs, with particular reference to comparative studies examining different serovars. Experimentally *Salmonella* Enteritidis is more often isolated from egg contents and seems to adhere better to reproductive tract mucosa, whilst *Salmonella* Typhimurium appears to provoke a more intense tissue pathology and immune response, and flock infections are more transient. However, it is observed that in many cases the present body of evidence does not identify clear differences between specific behaviours of the serovars Typhimurium and Enteritidis, whether in laying hens, in their eggs, or in the laying environment. It is concluded that further long-term experimental and natural infection studies are needed in order to generate a clearer picture.

## Introduction

*Salmonella* Typhimurium (ST) can be found in some laying flocks in the European Union (EU) (EFSA, 2007), including the UK (Snow *et al.*, 2007). In Europe, *Salmonella* infection of laying hens is dominated by *Salmonella* Enteritidis (SE) (EFSA, 2010a), and it is the predominant serovar isolated from eggs (De Buck *et al.*, 2004) and from egg-associated cases of human salmonellosis (EFSA, 2010a). In Australia, where SE has never been endemic in the national flock, ST is the principal cause of egg-associated salmonellosis outbreaks (OzFoodNet Working Group, 2009). Although often regarded as an external contaminant of eggs (EFSA, 2010b), ST has in earlier years been associated with outbreaks involving contamination of egg contents (Sesma *et al.*, 1987). This capacity of ST to infect laying flocks and to contaminate eggs may become more significant if the present trend of a declining prevalence of SE continues (HPA, 2010) and new egg-invasive strains of ST emerge. However, currently the observed risk for ST in eggs in the UK and EU is minimal, with other sources of ST (such as pig meat) being far more important (EFSA, 2009). The present review examines the existing knowledge regarding the features of ST infection of laying flocks and egg contamination, in comparison with other *Salmonella* serovars.

***Salmonella* Typhimurium in the laying hen.** *Findings in flocks naturally infected with Salmonella Typhimurium and other serovars.* Although ST is sometimes found in the environment of laying hens in the UK (Snow *et al.*, 2007), little work has been performed that examines the natural occurrence and distribution of the serovar at the level of the individual laying hen. Barnhart *et al.* (1991) examined pools of ovarian tissue taken at slaughter in the USA from spent flocks not associated with *Salmonella*

outbreaks. A wide range of *Salmonella* serovars was recovered, with between one and five being isolated from around three-quarters of flocks examined. However, neither ST nor SE was commonly isolated, being found in two and one of 42 flocks respectively. Whilst the possibility of surface contamination by extraneous serovars on the slaughter line cannot be excluded entirely, this nonetheless suggests that neither SE nor ST were commonly present in the ovarian tissue of these randomly-selected laying flocks.

*Experimental in vivo infections and comparisons with other serovars.* A number of studies have examined experimental ST infections of laying hens at various ages and by various routes, sometimes in comparison with other *Salmonella* serovars. In many such studies the aim has been to identify characteristics of colonisation and distribution within the inoculated hen that help to explain the pre-eminence of SE, compared with the other serovars examined, in contaminated eggs in many parts of the world. Findings have, in the main, proved to be frustratingly inconsistent. This may in part be because of variations in experimental approaches, including strains and inoculation routes.

*Intravenous inoculation studies.* In an early study (Baker et al., 1980) intravenous inoculation of mature laying hens with around  $5 \times 10^6$  colony-forming units (CFU) of an ST strain derived from a pheasant did not result in detectable contamination of faeces or eggs with the inoculated strain. Okamura et al. (2001a) also inoculated mature hens with around  $5 \times 10^6$  CFU *Salmonella*, this time with one of six serovars (Enteritidis, Typhimurium, Heidelberg, Hadar, Infantis or Montevideo). SE was the only serovar associated with clinical signs of depressed demeanour and feed intake, and caused the most prolonged bacteraemia. At post

mortem examination up to a week later, SE was recovered more frequently and in higher numbers than ST (or other serovars) at many sites including ovaries and the reproductive tract. Internal egg contamination was seen only with SE, but at low frequency (less than 10 % of eggs) compared with isolations from ovarian follicles and forming eggs.

By contrast, a higher intravenous dose ( $10^8$  CFU) of poultry isolates of serovars Enteritidis, Typhimurium, Heidelberg, Hadar, or Virchow in younger hens (22 weeks old) provided no evidence a week later of heavier colonisation of the spleen or reproductive organs by SE compared with ST (Gantois *et al.*, 2008). Both ST and SE showed generally better colonisation and yielded a higher frequency (40 to 80 %) of internally-contaminated eggs than did the other serovars in these young birds. This was a severe and unnatural challenge, with ST killing 29 % of birds and SE 8 % or 20 %, depending on the strain. Much lower mortality was observed with the other serovars.

**Oral and crop inoculation studies.** Infection by the oral route is a more natural presentation of Salmonella than intravenous administration. Oral inoculation of 36 mature hens with  $10^6$  CFU of one of ST, *Salmonella* Senftenberg and *Salmonella* Thompson for 10 consecutive days was not associated with contamination of the contents of any of the 232 eggs laid in this time (Cox *et al.*, 1973), or with recovery from viscera including ovaries after 10 days. However, almost all birds excreted the inoculated strains in faeces, and eggshell contamination rates of between 6.3 and 9.5 % of eggs were seen for all serovars.

In a short-term (four-day) study (Keller *et al.*, 1997), using young and mature laying hens inoculated with  $10^8$  CFU of one of three ST or three SE strains, both

serovars were observed to invade internal organs, oviduct and forming eggs to a similar degree, but only SE strains were isolated from laid eggs. Strain variation within serovar groups was observed.

Hassan and Curtiss (1997) administered to six- to 12-month-old hens an oral bolus of  $10^8$  CFU of either a ST strain virulent in young chicks or a SE strain associated with systemic invasion and egg contamination in hens. Gross pathology (including of the reproductive tract) was observed only among ST-inoculated hens but both serovars were frequently recovered from gastrointestinal, reproductive and other visceral samples for the following two weeks. Both serovars were also isolated frequently from the 181 eggs laid by the inoculated hens: 13% of yolks, 10% of albumen samples and 23 % of shells. Although ST was the less frequently isolated serovar from egg samples, the difference between the serovars was not statistically significant.

Experiments were performed using oral infection of point-of-lay pullets with  $10^7$  CFU of one of a number of ST definitive phage type 104 (DT104) or SE phage type 4 (PT4) strains (Williams *et al.*, 1998; Jørgensen *et al.*, 2000). Strains of both serovars showed tissue invasiveness and persistence in tissues for 14 days post inoculation, dependent on a functional *rpoS* (Sigma factor) locus. However, a ST DT104 strain showing environmental stress-sensitivity and *rpoS* mutation yielded similar egg contamination rates to ST strains that had intact *rpoS* loci and associated higher tissue invasiveness and persistence. In the same study, SE PT4 strains showed considerable diversity in tissue invasiveness.

Therefore, on present evidence the degree to which intestinal, hepatic, splenic, or reproductive tissues are colonized by ST or SE isolates following oral inoculation does appear to vary substantially. However, this variation has not been seen to



correlate with the likelihood of colonization of eggs forming in the oviduct (Keller *et al.*, 1997), or with the contamination of eggs after oviposition (Humphrey *et al.*, 1996; Keller *et al.*, 1997; Williams *et al.*, 1998; Jørgensen *et al.*, 2000).

It also appears that higher oral doses of ST are not associated with an increased likelihood of ST contamination of eggs. Using a virulent poultry ST strain and an oral dose of  $10^{10}$  CFU in point-of-lay and older hens, Brown and Brand (1978) observed substantial mortality and morbidity with frequent invasion of tissues, including ovaries, and variable depression of egg production. However, no contamination was detected among 257 eggs laid in the two to three weeks post-inoculation. Oral inoculation of around  $2 \times 10^8$  or  $2 \times 10^9$  CFU ST to mature laying hens resulted in faecal shedding but was not associated with contamination of any of 158 eggs (Baker *et al.*, 1980). Okamura *et al.* (2010) examined 10 ST strains from varied sources, inoculated orally in high numbers ( $10^8$  to  $10^{10}$  CFU) into mature and immature laying hens. A small proportion of eggs (11 of 3139) were internally contaminated, and only those from immature birds. There was no evidence of bacterial strain variation in internal egg contamination rates, but at this inoculation dose some strains were associated with depression of egg output whilst others were not.

This lack of a positive correlation between oral dose and likelihood of egg contamination is also seen in studies with SE, where there is even some evidence of an inverse relationship between bacterial dose and the likelihood of egg contamination. A low dose ( $10^3$  CFU) of SE PT4 given into the crop resulted in internally-contaminated eggs, whereas higher dose inocula were associated with morbidity and more marked humoral immune responses but no internal egg contamination (Humphrey *et al.*, 1991a). At the highest dose ( $10^8$  CFU), no

contamination of eggs, either of shell or contents, was seen. In another study, using crop inoculation of  $10^8$  CFU of SE strains into pullets followed by monitoring for two weeks, *Salmonella* was isolated in pure culture from only 2.5% of 441 eggs, despite the organism being isolated frequently from internal organs at *post mortem* examination (Humphrey *et al.*, 1996).

An oral inoculation study examining the potential role of a fimbrial operon (*peg*), which is present in SE but not ST, revealed that mutation of *pegA* reduced caecal colonisation of young (three week old) pullets, but only transiently (Clayton *et al.*, 2008).

**Other inoculation routes.** Breeding hens were inoculated by the vaginal route using semen artificially contaminated with SE or ST (Reiber *et al.*, 1991). Two weeks later the inoculated strain was recovered from the oviduct in 30 to 40 % of hens, and from the ovary in 20 %. Contamination (external only) was found on four to five percent of laid eggs. There was little difference between the serovars in these respects.

Miyamoto *et al.* (1997) examined internal dissemination and egg contamination at up to seven days following administration of between  $10^6$  and  $10^7$  CFU of a single strain of SE by vaginal and cloacal routes to mature layer hens. By contrast with intravenous administration, these routes were associated with much less morbidity and did not yield isolations from the ovaries or upper reproductive tract (infundibulum and magnum); invasion was however seen in the liver, spleen and lower reproductive tract. Intravaginal inoculation resulted in *Salmonella* isolation from eggs laid by around 50% of hens, both in contents and on shells. Cloacal inoculation was associated with isolations from eggshells but not egg contents.

Direct inoculation by aerosol, with an estimated delivered dose of  $10^2$  or  $10^4$  CFU ST DT104, readily infected point-of-lay pullets systemically and, for the two-week duration of the study, was associated with a substantially higher frequency of internally-contaminated eggs than was observed following oral inoculation of a higher dose ( $10^7$  CFU) of the same ST strain (Leach *et al.*, 1999). By comparison, a similar dose of SE PT4 delivered by aerosol to birds of a similar age to the above was associated with systemic invasion, including of the reproductive tract, but no detectable egg contamination (Baskerville *et al.*, 1992). In both studies, higher aerosol doses ( $10^3$  to  $10^5$  CFU) were associated with morbidity.

Natural exposure of breeding hens to SE via inoculated seeder pen-mates is sufficient to generate *Salmonella*-positive eggs (Cox *et al.*, 2000), but similar studies for ST are lacking.

**In vitro studies.** Various *in vitro* studies have pursued the hypotheses that SE is at a comparative advantage to ST (and other serovars) in its capacity for egg contamination owing to features that enhance invasion or survival in key tissues or in the forming egg.

SEF-14 fimbriae, encoded by SE and other type D salmonellas but not ST, have been investigated as a potential adhesin and/or invasion factor, both *in vivo* in the avian and murine intestine and other viscera, and also *in vitro* in avian ovarian granulosa cells and macrophages plus standard enteric and other epithelial cell lines (Peralta *et al.*, 1994; Ogunniyi *et al.*, 1997; Rank *et al.*, 2009). Some effects, including adhesion to granulosa cells (Thiagarajan *et al.*, 1996), and persistence in avian liver and spleen (Rajashekara *et al.*, 2000) have been attributed to SEF-14. However, these studies have not provided firm evidence of a significant role in adhesion or invasion

for SEF-14 in wild-type SE. Nonetheless, SEF-14 may yet be shown to assist SE in some tissues at certain stages of infection.

ST showed more resistance to killing by avian macrophages and induced more macrophage membrane changes and interferon- $\gamma$  production than did SE (Okamura *et al.*, 2005). However, ST and SE strains were similar in respect of their ability to invade isolated ovarian follicles at various stages of development (Howard *et al.*, 2005).

Investigations using *in vitro* organ culture of vaginal epithelium from mature laying hens showed that two of three tested SE strains adhered to and invaded the epithelium significantly more avidly than did three tested ST strains (Mizumoto *et al.*, 2005). Indeed, several serovars (Enteritidis, Typhimurium, Agona, Heidelberg, Hadar, Infantis and Montevideo) could be ranked in terms of adherence and invasiveness in this test in a manner that correlated with surface lipopolysaccharide type and also with the frequency of egg-associated outbreaks of salmonellosis associated with each serovar.

In a study of poultry isolates of various *Salmonella* serovars inoculated to a final concentration of  $10^2$  to  $10^3$  CFU/ml in albumen, then incubated at or near avian physiological temperature, ST showed significantly better survival at 42 °C than SE or *Salmonella* Heidelberg, and these in turn survived significantly better than the non-egg-associated serovars Virchow and Hadar (Gantois *et al.*, 2008). In egg albumen incubated at 37 °C with around  $10^3$  CFU/ml inoculated *Salmonella* organisms, the average survival time of 10 (non-egg-associated) ST strains was, by contrast, significantly shorter than that of 15 SE strains (Clavijo *et al.*, 2006). It was postulated from genetic analyses that gene regulation, rather than the presence or absence of certain genes may be the most significant factor promoting survival of *Salmonella* in

this environment. The lipopolysaccharide 'O' antigen biosynthesis gene *rfbH* appears to be an important factor in the survival of SE in egg albumen at avian physiological temperature (Gantois *et al.*, 2009), but comparisons with ST in this respect have not been reported.

Alongside these conflicting findings, a similar experiment compared the survival of several SE and ST DT104 strains that were inoculated (at  $10^3$  CFU/ml) into egg albumen and then incubated at either 42 °C or 37 °C, and showed no significant differences in rates of decline of the bacteria (Guan *et al.*, 2006). The experimental doses of *Salmonella* reported in these studies are orders of magnitude higher than the typical concentrations of *Salmonella* found in the albumen of laid eggs from naturally or experimentally infected hens (Humphrey *et al.*, 1991b; Gast *et al.*, 2002). The antibacterial properties of albumen may be overcome by high numbers of contaminants and /or trace amounts of iron (Schoeni *et al.*, 1995; Kang *et al.*, 2006), which may go some way to explaining the inconsistent results from these varied models for the fate of *Salmonella* contaminants in the forming egg.

***Salmonella Typhimurium in the laid egg.*** *Surveys and examinations of commercially produced eggs.* Although current culture techniques may not strictly separate external from internal contamination (FSA, 2004), the *Salmonella* serovars isolated from shell surfaces are diverse, whereas internal contamination from intact eggs is dominated by SE (De Buck *et al.*, 2004). In Australia, where SE is not present in layer flocks, ST is principally regarded as an external contaminant of eggs (EFSA, 2010b).

*Experimental studies involving hens.* Despite evidence for the preponderance of external contamination and the rarity of internal contamination among eggs yielding ST (De Buck *et al.*, 2004; EFSA, 2010b), experimental oral infections with a variety of ST strains have produced many instances of internally contaminated eggs (Hassan & Curtiss, 1997; Williams *et al.*, 1998). In addition, experimental studies have found little or no correlation between the detection of SE or ST in hens' faeces and their isolation from eggs laid by the same individuals (Humphrey *et al.*, 1991a; Gast & Holt, 1998; Williams *et al.*, 1998; Okamura *et al.*, 2010), suggesting that faecal surface soiling of eggs may be a relatively unimportant route for the contamination of eggs with ST in these admittedly short-term experiments.

After intravaginal inoculation of hens, SE was isolated from eggs significantly more frequently than was ST or any of the other four serovars (Heidelberg, Hadar, Infantis and Montevideo) tested (Okamura *et al.*, 2001b). ST was the only serovar other than SE to be isolated from egg contents. SE was most frequently isolated from the inner aspect of the eggshells suggesting that, in this model at least, SE may have an enhanced ability to localise to this area, either by deposition as membranes form in the oviduct (an area most often colonised by SE in this study also) or by subsequent penetration of the shell. The inner shell membranes may be a relatively privileged site, protected both from external desiccation and from antibacterial elements in the albumen (Berrang *et al.*, 1999). Contamination of the contents of eggs by ST has been reported following experimental infection of hens and pullets via intravenous (Gantois *et al.*, 2008), oral (Hassan & Curtiss, 1997; Williams *et al.*, 1998; Okamura *et al.*, 2010) and aerosol (Leach *et al.*, 1999) routes, as described in previous sections. In those studies where both SE and ST have been examined, ST has appeared to cause contamination of egg contents at a similar or lower frequency compared with SE.

Experimental studies with eggs. SE is typically present in the albumen of naturally contaminated eggs, in low numbers of less than 10 CFU to (more rarely) hundreds of CFU per egg (Humphrey *et al.*, 1991b). When about 10 CFU of egg-associated and non-egg-associated serovars (SE, ST, Senftenberg, Stanleyville, Mbandaka, Blockley) were inoculated into the albumen of eggs up to three weeks old and held at 20 °C, slow multiplication was documented, with some strain variations, but there were no clear differences in this respect between the serovars (Messens *et al.*, 2004). Similarly, little difference was observed between the growth rates in albumen or yolk of SE, ST or *S. Heidelberg* inoculated into eggs in higher numbers ( $10^2$  or  $10^4$  CFU) and held at 4 °C, 10 °C, or 25 °C (Schoeni *et al.*, 1995). When bacterial cells penetrate the vitelline membrane and invade the yolk of fresh eggs, multiplication to much higher numbers occurs. This requires motility if the bacterial cells are initially in the albumen or shell membranes. Two ST isolates proved to be as successful as SE at this process following inoculation of the albumen with fewer than 10 bacterial cells (Cogan *et al.*, 2004). The production of thin aggregative (curli) fimbriae is associated with an enhanced capability to invade the yolk and grow to high densities. Cogan *et al.* (2004) found ST and SE to be similar in this respect.

Both shell and shell membranes constitute significant barriers for *Salmonella* penetration of eggs (Östlund, 1971). The penetration of the shells of laid eggs has been examined for many *Salmonella* serovars, including ST. In a comparison not including SE, ST was consistently and significantly better than 10 of 11 other serovars at rapidly penetrating warm eggs immersed in bacterial suspensions (Sauter & Petersen, 1974). ST showed a marginal advantage over SE in a similar study, using cooler eggs (Miyamoto *et al.*, 1998). The rapid exposure of freshly-laid eggs to a high

density of ST by dry contact ( $10^6$  CFU/g bedding) was associated with a high proportion of ST-positive eggs following 19 day's incubation (Padron, 1990).

The egg cuticle is a hydrophobic, proteinaceous outer layer, coating the shell and occupying pores, that dries and hardens soon after oviposition. However, it does not consistently cover the whole egg surface and its role in resisting penetration by *Salmonella* is therefore uncertain (Messens *et al.*, 2005). Using two-day-old eggs, Williams *et al.* (1968) demonstrated that ST applied in avian faeces will penetrate to the inner surface of the shells of a minority of eggs within minutes at room temperature. This effect was enhanced if areas of high shell permeability (shown by the uptake of food dye) were targeted for exposure. It was concluded that external warmth and increased moisture aided penetration, but that shell thickness was not significant and the challenge in terms of bacterial numbers was relatively unimportant.

### ***Salmonella* Typhimurium infection and persistence in laying flocks**

SE was found in three and a half times as many UK layer holdings than ST in a recent systematic survey (Snow *et al.*, 2007). However, in contemporaneous surveys of shell eggs in the UK, dominated by UK-laid eggs (FSA, 2004, 2007), SE accounted for 13 of 16 positive samples and ST was not isolated from any sample. This may reflect the fact that the largest holdings were nearly six times more often positive for SE than for ST. However, it is also possible that the layer house environment may contribute to differences between the frequencies of *Salmonella* serovars in shell eggs.

The persistence of SE in a layer house has been shown to be positively associated with the level of rodent activity in the house, but this strong correlation with rodents was not observed for ST (Carrique-Mas *et al.*, 2009). Rodents, and mice



in particular, are a very common problem in laying houses and correlations with persistent SE infection of flocks have been observed by several workers (Henzler & Opitz, 1992; Guard-Petter *et al.*, 1997; Garber *et al.*, 2003). It has been theorised that SE may derive benefit via enhancement of cell wall lipopolysaccharide for persistence or invasion (Guard-Petter, 2001) following passage through henhouse rodents.

Rodents also provide an opportunity for multiplication of *Salmonella* in the henhouse (Henzler & Opitz, 1992; Wales *et al.*, 2006), which may differ between SE and other serovars. The oral virulence of SE strains in mice is variable (Poppe *et al.*, 1993; Ekawa *et al.*, 2009) but frequently much lower than for ST, which typically carries a large plasmid that confers virulence in mice (Helmuth *et al.*, 1985; Baggesen *et al.*, 1992). Consequently, extended excretion by mice may not occur as frequently with ST as with SE.

It might be hypothesised that chicken genetics favour the establishment and maintenance of SE rather than ST in flocks. Differential susceptibilities of chicken genetic lines to *Salmonella* infection have been observed and appear to be multifactorial, in part involving various aspects of the function of macrophages and other immune cells (Wigley, 2004). However, on the present limited evidence it appears that genetic resistance to acute or chronic infection by one of these serovars is also associated with resistance to the other (Calenge *et al.*, 2010), but systematic comparative studies in this area are lacking.

There are many sources of ST for humans; in the EU pig meat, dairy products, companion animals, wild animals and environmental contamination are considered to be far more important sources than eggs (EFSA, 2010b; Pires *et al.*, 2010). In the UK and some other countries the increase in free-range egg production has led to a greater risk of exposure of laying hens to ST strains from wild birds. Many of these strains

appear to be host-adapted (Rabsch *et al.*, 2002) and do not pose a public health threat to chicken egg production (EFSA, 2010b). Infection of such strains of ST in chicken flocks tends to be short-lived compared with SE infections (Carrique-Mas *et al.*, 2009), with no evidence of egg transmission even in breeding flocks where eggs are incubated at 37 °C for hatching (Littrup *et al.*, 2010). In contrast to poultry and pig meat sectors, where there is no statutory restriction of sales of product when ST is found on the holding, egg sales are restricted for the whole of the life of the flock if ST is found during monitoring of a laying flock even if the infection does subsequently clear. It is important for egg producers to control sources of ST as effectively as possible. Predominant sources include wild birds, contaminated feed, pigs and cattle, companion animals, rodents and hatchery contamination (Refsum *et al.*, 2002). The risk from such sources can be reduced, but not totally eliminated, by good biosecurity and farm hygiene procedures.

## Summary

ST has an established ability to be transmitted to humans via shell eggs, but in most parts of the developed world, including the UK, it is currently much less significant in this role than is SE. However, the reasons for this are still poorly understood and much of the survey and experimental data comparing ST with other serovars is inconsistent, conflicting, or not illuminating. In addition, there is very little useful published data derived from field studies, natural infections, or long-term experiments. Large variations are observed between many of the superficially similar studies reported, in terms of methodology employed, morbidity, systemic colonisation

and frequencies of egg contamination. The differing findings cannot easily be attributed simply to differences in doses or inoculation routes. It may be that variations in experimental *Salmonella* strains, observed for SE and ST in studies cited in the present review (Keller *et al.*, 1997; Williams *et al.*, 1998) are responsible for apparently inconsistent findings, and further studies in this area are needed, particularly for non-Enteritidis serovars. There may also be variation attributable to genetic differences in chicken lines.

Experimental studies comparing serovars confirm that SE is consistently more frequently found as an internal contaminant of eggs than other serovars, including ST. Theories regarding the clear advantages displayed by SE in egg contamination have tended to focus on its ability to colonise the chicken ovary and reproductive tract, and thereby potentially to contaminate eggs at many stages of formation (De Buck *et al.*, 2004). The fact that many *Salmonella* serovars appear to have poorer capabilities than SE in respect of internal colonisation of laying hens, lends support to this theory. However, there does not appear to be a consistent difference between SE and ST in this respect, in the studies reported. Indeed, even between ST strains, substantial variation in systemic colonisation capability has been observed, which did not correlate with the observed egg contamination frequencies. However, most experimental studies in this area have been short-term (up to about two week's duration) and have used high infective doses. These conditions may minimise or fail to reveal differences between serovars in terms of infectivity over the whole production cycle.

Specific examinations of putative colonisation factors and colonisation sites (intestine, ovary, reproductive tract) have not yet yielded any strong evidence of consistent differences between ST and SE, although some work suggests SE may be

able to adhere especially well to reproductive tract mucosa and to colonise associated glandular tissue. No convincing correlation at the individual hen level between the isolation from faeces of ST or SE and isolations from eggs has been found, suggesting that contamination of forming eggs within the ovary and oviduct is the key factor that determines the rate of egg contamination. On present evidence, there do not appear to be consistent differences between ST and SE in respect of their ability to penetrate eggshells, to survive in albumen at physiological or storage temperatures, or to penetrate the vitelline membrane and colonise the yolk of formed eggs. It should be noted, however, that in many of these areas ST and SE do appear to outperform many non-egg-associated *Salmonella* serovars, thereby suggesting that capability in these matters may be necessary but not sufficient alone to enhance *Salmonella* contamination of commercially produced eggs.

An area where there may be a more consistent difference between SE and ST is in the propensity of a serovar to generate pathology and/or to provoke a strong immunological response in the host. Some authors have speculated that the typically rather benign effect of SE on its avian host, compared with the more pathological consequences of ST infection (including in the reproductive tract) may assist the invasion of reproductive tissue and forming eggs by SE after its avoidance of the local cellular immune mechanisms (Guard-Petter, 2001; De Buck *et al.*, 2004). Findings of increased pathology in ST versus SE infections have been reported by some workers cited in the present review (Hassan & Curtiss, 1997; Okamura *et al.*, 2005), and greater cross-protection has been observed following vaccination with ST than with SE (Gast, 2007). These observations both lend some support to the hypothesis that ST is likely to provoke a stronger and more rapid immune response than SE and therefore be more limited in its progress and cleared from the infected bird more quickly.

Explanatory factors may eventually emerge to clearly distinguish between *Salmonella* strains with differing propensities to contaminate eggs. For the present, based on *in-vivo* challenge studies, some strains of ST appear to have similar capabilities to SE in respect of intestinal colonisation and systemic infection of laying hens, survival in the forming and laid egg, and penetration of eggshells and membranes. If the main difference lies in the ability of SE to cause persistent colonisation of the ovary and oviduct then experiments using natural infection routes and doses, plus long-term monitoring and field investigations, will be needed to demonstrate this in relation to the public health risk. Such studies are, at present, lacking.

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**General comments:**

This manuscript reviews the present knowledge on the significance of Salmonella Typhimurium infection in layers with special regard of pathogenicity and egg infection, in comparison with other serovars, especially with S. Enteritidis.

This is a very important and relatively novel aspect regarding that so far, the utmost attention has been focused on S. Enteritidis, although S. Typhimurium is recognized as the second most important regulated Salmonella serovar for layers (EFSA-Opinion 2010). The task is quite difficult regarding the large number of papers published in the area of Salmonella in layers and eggs, and the authors have presented a good basis for our “fresh look” at this subject.

The present reviewer has the impression that the authors have given more emphasis to those data which are indicating less differences between S. Enteritidis and S. Typhimurium in terms of infection of reproductive organs and eggs. At the same time they do not provide their own synthesis for the pathogenetic significance of S. Typhimurium in layers and in egg contamination in comparison with S. Enteritidis. This attempted to be a systematic review of the available published evidence, focusing on studies of S. Typhimurium, including the many comparative studies. We don't believe that we have given undue emphasis to studies that minimise differences between serovars Typhimurium and Enteritidis – it's just that (as discussed in the Summary) the present body of data on the survival and behaviour of ST in hens and eggs does not adequately explain the observed epidemiological differences between SE and ST.

We feel that, given this clear gap in understanding, attempts at an explanatory synthesis using the existing data should be restricted to observations on areas where promising differences between the serovars have been found, plus suggestions as to useful avenues for further research. We believe that we have provided this in the Summary and Abstract.

This review could be made more effective if the authors would try to give their opinion based on more consideration of data that speak for the central role of S. Enteritidis in contamination of eggs.

The central role of S. Enteritidis in eggs is acknowledged in the Introduction and Summary sections, but this is a review with S. Typhimurium as its focus. Given the available evidence, it is not clear why Typhimurium is so much less significant as an egg contaminant than Enteritidis and this is one of the principal themes of the Abstract and Summary.

**Some suggestions:**

1. It would help to have a short “Table of contents” listing the titles of chapters and subchapters of this Review. **Added**
2. Based on the main comment above, the Abstract could be re-arranged, a bit corrected, and finished with a conclusive statement (suggested version attached below). **Amended in line with suggestion.**

3. It would make this Review more effective if there would be a “Conclusion” after the “Abstract”
4. A few sentences in short paragraph could be given about the significance of “genetic resistance” to Salmonella Typhimurium of certain chicken lines (in order to make some basis for mentioning it in the Summary) - [Paragraph added in section on flock infections](#)
5. P5, L20-25: This paragraph could be considered for omission as it is not bearing a comparative value to S. Typhimurium infection. [Deleted](#)
6. “Strain variations” within the same serovar are mentioned occasionally (i.e. P6, L14, and P7, L9). However, this reviewer feels that this aspect has been scarcely investigated in the literature especially for non-SE serovars, and the need for more studies on this aspect could be emphasized a bit more, because such studies could help explaining some of the inconsistencies between some results. [Comment and citations added in Summary](#)
7. The role of SEF14 fimbriae as a potential adhesin contributing to colonization of reproductive tissues by S.E. is a bit “played down” by the interpretation of the authors. It is believed that they should be more analytic when reviewing the data in the literature and not just conclude about data for the wild-type SE (P10, L14 -19). [Paragraph rewritten and references added.](#) Furthermore they could mention the role of “Curly fimbriae” in eggs (Cogan et al., 2004). [Added to “Experimental studies with eggs” section.](#)
8. The paragraph about S. Typhimurium in duck eggs (P16., L10-14) does not seem necessary for this review. It is quite well known that eggs from waterfowl are frequently contaminated with ST. [Deleted](#)
9. P6, L3-8: regarding the possible difference in role of rodents for persistence of SE in a henhouse, in contrast to ST, it may be worth considering that mice are much more susceptible to ST. As a consequence, shedding of ST by mice may be less frequent. [Comments and references added in laying flocks section](#)

### **Suggested re-arrangements and changes in ABSTRACT**

.....“To that end, the present review examines the published literature on Salmonella Typhimurium in laying hens and eggs, with particular reference to comparative studies examining different serovars., Experimentally Salmonella Enteritidis is more often isolated from egg contents and seem to adhere better to reproductive tract mucosa, whilst Salmonella Typhimurium appears to provoke a more intense tissue pathology and immune response, and as a consequence, flock infections are more transient. There is, however, a lack of data that arise from long-term natural or experimental infection studies. It is observed that in many cases the present body of data does not identify clear differences between specific behaviours of the serovars Typhimurium and Enteritidis, whether in laying hens, in their eggs, or in the laying environment. It is concluded that further long term, and natural infection studies are needed in order to generate a clearer picture.”