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A REVIEW

Colonization of the chicken reproductive tract and egg contamination by *Salmonella*

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1. SUMMARY

The food-borne salmonellosis pandemic in humans is for a large part caused by the consumption of contaminated eggs. Infection of the reproductive organs of laying hens often is the underlying phenomenon leading to the production of contaminated eggs. To date, the pathogenesis of reproductive tract infection in hens has not received the full attention it merits in relation to its importance in transmitting *Salmonella* infections within the poultry population and from poultry to man. This review discusses the different possible infection routes leading to egg contamination and emphasizes on the oviduct and ovary colonization in the process of egg contamination. The role of known bacterial virulence factors in the pathogenesis of reproductive tract infection is discussed. Immune responses in the oviduct, related to *Salmonella* infection, are described. Finally, different possible approaches to protect laying hens against reproductive tract infection by *Salmonella* are reviewed.

2. INTRODUCTION

The number of *Salmonella* Enteritidis outbreaks in humans has dramatically increased throughout the world since the mid to late 1980s (Hogue *et al.* 1997). The incidence of other *Salmonella* serovars has worldwide remained the same or has declined (Olsen *et al.* 2001; van Duijkeren *et al.* 2002; Cogan and Humphrey 2003). Epidemiological analyses point to eggs and egg products as the major risk factors for *S.* Enteritidis infection in humans (St.Louis *et al.* 1988; Hedberg *et al.* 1993).

Most reported *Salmonella* outbreaks in humans in the last 10 years in the world, of which the origin of infection was traced back to eggs, were caused by *S.* Enteritidis; some *S.* Typhimurium and one *S.* Heidelberg isolate are rare exceptions (Table 1). Serotypes other than Enteritidis indeed are isolated from egg contents at a much lower frequency (Indar *et al.* 1998; Okamura *et al.* 2001b).

The percentage of naturally *Salmonella*-infected eggs varies in different public health laboratory reports. The results of several bacteriological analyses of eggs in the UK are shown in Table 2.

In a naturally infected layer flock the proportion of infected eggs that are laid varies (Humphrey 1989). Most studies show the percentage to be below 0·03% (Kinde *et al.* 1996; Ebel and Schlosser 2001). In artificially infected hens the percentage can range from 0 to 27·5% (Keller *et al.* 1995; Okamura *et al.* 2001b).

In a 1995 report, 191 eggs were contaminated with *S.* Enteritidis out of 738 000 eggs tested, whereas *S.* Typhimurium was isolated from only one egg (Anon. 1995a). However, in an experimental setup egg contents

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Table 1 Overview of *Salmonella* serotypes isolated from outbreaks in humans, with eggs as the presumed origin, in the last decade all over the world; SE: *S.* Enteritidis, ST: *S.* Typhimurium, SH: *S.* Heidelberg

Publication date	Isolates	Serotype	Phage type	Place	Reference
2003	1	SE	PT5	Austria	Berghold *et al.* (2003)
2002	1	SE	/	Denmark	Locht *et al.* (2002)
2002	1	ST	PT135	Australia	Sarna *et al.* (2002)
2002	1	ST	PT135	Australia	Tribe *et al.* (2002)
2002	1	ST	PT135	Australia	Hall (2002)
2001	1	SE	/	Japan	Dohtsu *et al.* (2001)
2000	4	SE	/	US	Anon. (2000)
2000	1	SE	/	Italy	Lopalco *et al.* (2000)
1999	1	SE	/	Japan	Osaka *et al.* (1999)
1999	1	SE	/	US	McNeil *et al.* (1999)
1999	1	SE	PT6	Denmark	Neimann *et al.* (1999)
1999	1	SE	PT4	UK	Wilson *et al.* (1999)
1998	1	SE	PT4	Italy	Nastasi *et al.* (1998)
1998	5	SE	PT4	Brazil	Peresi *et al.* (1998)
1998	1	SE	PT6	UK	Dodhia *et al.* (1998)
1998	12	SE(11), SH(1)		Italy	Petersen and James (1998)
1997	1	SE	PT4	N. Ireland	Doherty *et al.* (1997)
1997	10	SE	/	Mexico	Molina-Gamboa *et al.* (1997)
1997	1	ST	/	Spain	Carraminana *et al.* (1997)
1996	2	SE	/	US	Koo *et al.* (1996)
1996	1	SE	PT4	UK	Evans *et al.* (1996)
1996	1	SE	PT4	US	Boyce *et al.* (1996)
1996	1	SE	PT4	UK	Wight *et al.* (1996)
1995	1	SE	PT4	UK	Bates and Spencer (1995)
1995	1	SE	PT4	UK	Anon. (1995b)
1995	1	SE	/	Brazil	Kaku *et al.* (1995)
1995	1	SE	PT6	UK	Brugha *et al.* (1995)
1994	1	SE	PT4	UK	Morgan *et al.* (1994)

were almost equally frequently infected when an oral challenge of laying hens with *S.* Typhimurium was compared with *S.* Enteritidis (Hassan and Curtiss 1997). In another study, oral nor i.v. challenge of laying hens with *S.* Typhimurium resulted in the contamination of eggs (Baker *et al.* 1980). However, experimental infection with *S.* Typhimurium DT104 can lead to the contamination of intact eggs (Williams *et al.* 1998; Leach *et al.* 1999).

There seems to be a link between egg contamination and the infection of the laying hen, as *S.* Enteritidis is far more frequently isolated from naturally infected hens than any other serovar (Anon. 2003). The ability of *S.* Enteritidis to colonize the reproductive organs may be a selective advantage over other serotypes (Keller *et al.* 1997) and may be one of the reasons that egg contamination with *S.* Enteritidis has increased (Okamura *et al.* 2001b). Understanding the *S.* Enteritidis-specific factors involved in the egg contamination process should be the basis for the development of control measures.

3. EGG CONTAMINATION

Egg contamination by *S.* Enteritidis can be caused by penetration through the eggshell from contaminated faeces after or during oviposition (Gast and Beard 1990b; Barrow and Lovell 1991; Humphrey *et al.* 1991b) or by direct contamination of yolk, albumen, eggshell membranes or eggshells before oviposition originating from the infection of reproductive organs with *S.* Enteritidis (Timoney *et al.* 1989; Shivaprasad *et al.* 1990).

3.1 Surface contamination of eggs and penetration through the eggshell

A wide range of serovars has been recovered from eggshells (de Louvois 1993b), including *S.* Enteritidis (Poppe *et al.* 1992; de Louvois 1993b; Humphrey 1994; Schutze *et al.* 1996). The presence of many different *Salmonella* serotypes on the surface of the shells of eggs represents a potential threat to public health, just as well as contamination of the contents of the egg. Surface contamination however may be the result of either infection of the lower reproductive tract or faecal contamination. Faecal contamination is unlikely to occur during oviposition in a healthy laying hen. Indeed, when a healthy hen lays an egg, its bearing everts the vagina beyond the alimentary tract. This protects the emerging egg from faecal contamination. In addition, the stretching of the

Table 2 Percentages of *Salmonella* infected eggs in different public health laboratory reports

Institute	Year	Country	Type of sample	Serovar	Ratio positive (%)	Reference
PHLS	2002	UK	Egg pools	All serovars	30/407 (7·4)	Mitchell *et al.* (2002)
PHLS	1991	UK	Egg content	*S.* Enteritidis	34/5700 (0·6)	Humphrey *et al.* (1991b)
				Other serovars	0/5700 (0·0)	
			Egg content	*S.* Enteritidis	18/1952 (0·9)	
			Eggshells	*S.* Enteritidis	21/1952 (1·1)	
PHLS	1993	UK	Complete eggs	All serovars	1/650 (0·15)	de Louvois (1993a,b)
				S. Enteritidis	1/850 (0·1)	
		UK/imported		All serovars	1/370 (2·7)	
				S. Enteritidis	1/2720 (0·04)	
ACMSF	1995/'96	UK	Egg pools	All serovars	138/13970 (1·0)	Anon. (2001)
				S. Enteritidis	133/13970 (1·0)	
				S. Enteritidis PT4	82/13970 (0·6)	
ACMSF	1996/'97	UK	Egg pools	All serovars	29/1433 (2·0)	Anon. (2001)
				S. Enteritidis	18/1433 (1·3)	
				S. Enteritidis PT4	2/1433 (0·1)	

PHLS, Public Health Laboratory Service, Exeter, UK; ACMSF, Advisory Committee on Microbiological Safety of Foods.

cloacal lining effectively makes the intestinal tract somewhat slit-like, further reducing the opportunity for contamination of eggshell. This is why most eggshells in healthy birds are not covered in faeces at oviposition. Faecal contamination may however very well take place in the environment after oviposition. If contamination through contact with faeces or the environment is important, then the hygiene in the chicken house and during egg handling and processing is critical.

Penetration of eggshell by *S.* Enteritidis (Haigh and Betts 1991; Dolman and Board 1992; Schoeni *et al.* 1995; Miyamoto *et al.* 1998; Wang and Slavik 1998), as well as *S.* Typhimurium (Padron 1990; Schoeni *et al.* 1995; Berrang *et al.* 1998; Miyamoto *et al.* 1998; Berrang *et al.* 1999) and other serovars (Javed *et al.* 1994) has repeatedly been described under experimental conditions. These exclusively experimental penetration assays have lead to the hypothesis that the contents of eggs can become contaminated immediately after laying through pores or cracks in the shell. However, somehow this penetration of *Salmonella* bacteria does not seem to occur at the same rate in practice, as the spectrum of *Salmonella* serovars isolated from the egg surface does not correspond with that found in the egg contents, the latter being almost uniquely *S.* Enteritidis. Experimental surface contamination by *E. coli* can also lead to the contamination of egg contents (Haigh and Betts 1991).

Only few reports suggest that egg contents are more likely to become contaminated during passage through the cloaca than as a result of ovarian infection (Rodrigue *et al.* 1990; Barrow and Lovell 1991). It is however impossible to discriminate between surface contamination from the environment and contamination during formation of the eggs, when total eggshells are cultured. Genuine egg surface contamination could be differentiated from shell and shell membrane contamination that took place inside the reproductive tract, by dipping eggs in culture broth before their surface is sterilized and the eggshells are cultured, like has been done in previous reports (Bichler *et al.* 1996; Miyamoto *et al.* 1997; Okamura *et al.* 2001a,b).

3.2 Contamination of eggs during egg formation

As already mentioned above, *S.* Enteritidis is the dominant serotype isolated from egg contents (Paul and Batchelor 1988; Perales and Audicana 1988; Humphrey 1989; Mawer *et al.* 1989). An inconsistent relationship exists between *S.* Enteritidis contamination of the eggshell and that of the egg content (Humphrey 1989; Mawer *et al.* 1989; Humphrey *et al.* 1991c; Methner *et al.* 1995). This indicates that contamination of egg contents is more likely to take place in the reproductive organs than by eggshell penetration. Examination of eggs from birds infected artificially found no relationship between faecal carriage of *S.* Enteritidis and the presence of the bacterium in egg contents (Gast and Beard 1990a; Humphrey *et al.* 1991b). It is also possible to isolate *S.* Enteritidis PT4 from the reproductive tissue of infected hens, in the absence of intestinal colonization (Lister 1988; Bygrave and Gallagher 1989; De Buck *et al.* 2004b).

S. Enteritidis has been found in both the yolk and albumen of eggs laid by infected hens (Humphrey 1989; Timoney *et al.* 1989; Shivaprasad *et al.* 1990; Humphrey *et al.* 1991c; Keller *et al.* 1995; Bichler *et al.* 1996). Albumen is the compartment most frequently contaminated by *S.* Enteritidis, according to most authors (Gast and Beard 1990a; Shivaprasad *et al.* 1990; Humphrey *et al.* 1991c; Gast and Beard 1993; Humphrey 1994; Methner *et al.* 1995; Price

et al. 1995; Anon. 1998, 1999), although the incidence of yolk contamination has been reported by Bichler *et al.* (1996) and by some more recent studies of Gast and Holt (2000) and Gast *et al.* (2002) to be greater than the incidence of albumen contamination. Yolk contamination points to the ovary as site of origin of the egg contamination.

Contamination of the albumen by *S.* Enteritidis is believed to occur during passage of the egg through the oviduct (Gast and Beard 1990b; Shivaprasad *et al.* 1990; Humphrey *et al.* 1991c; Hoop and Pospischil 1993; Reiber and Conner 1995). Several studies even suggest that *S.* Enteritidis most frequently migrates into chicken eggs through the upper oviduct in association with albumen (Gast and Beard 1990a; Shivaprasad *et al.* 1990; Hoop and Pospischil 1993; Humphrey 1994; Keller *et al.* 1995). *S.* Enteritidis has been found in association with secretory cells of the upper and lower magnum by immunohistochemical staining (Hoop and Pospischil 1993). This is also compatible with the hypothesis that the pathogen may contaminate forming eggs through the albumen.

Keller *et al.* (1995) observed a higher contamination rate of forming eggs as compared with laid eggs. They suggested a heavier colonization of the eggs during their development, diminished by factors within the eggs, such as antibodies, antibacterial enzymes, iron-sequestering and bacterial protease-inhibiting proteins, controlling the pathogen before the eggs are laid.

Egg shell and eggshell membrane are produced in the lower reproductive tract. These compartments of the egg also may be contaminated during egg development. Contamination of eggshells and eggshell membranes by *S.* Enteritidis have been reported to occur frequently (Humphrey 1989; Humphrey *et al.* 1991c; De Buck *et al.* 2004b). In some studies it is even reported as the most infected site of contaminated eggs (Bichler *et al.* 1996; Miyamoto *et al.* 1997; Okamura *et al.* 2001b). However, as *Salmonella* bacteria can penetrate eggshells, it is difficult to distinguish between contamination during formation of the egg or after oviposition. Still, localization on the inner side of eggshells would put the bacteria in a favourable position, as the bacteria may be more or less protected from the antimicrobial factors in the egg white. The limiting membrane on the inner side of the eggshell membranes retains the antimicrobial factors of the albumen inside the egg (Tung and Richards 1972). In hatching eggs, the bacteria situated in the shell membranes may not infect the embryo until late during incubation. Indeed, infection may be delayed until pipping.

4. COLONIZATION OF THE OVARY

A systemic *S.* Enteritidis infection in laying hens can lead to the colonization of the ovary or the oviduct (Keller *et al.* 1995; Miyamoto *et al.* 1997; Okamura *et al.* 2001a,b; De Buck *et al.* 2004a). Both organs can be infected independently from each other (Kinde *et al.* 2000), at the same time or maybe one after the other. The concept of transovarian transmission of *S.* Enteritidis is generally accepted (Shivaprasad *et al.* 1990; Thiagarajan *et al.* 1994).

Following experimental oral inoculation of laying hens with *S.* Enteritidis, bacteria are isolated from the tissue layers surrounding the yolk in preovulatory follicles (Thiagarajan *et al.* 1994). These findings indicate that *Salmonella* can interact with the cellular components of the preovulatory follicle. Indeed, *S.* Enteritidis has been shown to interact with granulosa cells in a specific manner and to invade and multiply in these cells (Thiagarajan *et al.* 1994, 1996a). It is even suggested that the granulosa cell layer of the preovulatory follicles may be a preferred site for the colonization of the chicken ovary by invasive strains of *S.* Enteritidis.

A haematogenous spread to the ovary has been suggested to occur (Timoney *et al.* 1989; Shivaprasad *et al.* 1990). Blood-borne organisms may be deposited near the basement membrane of the theca cells (Thiagarajan *et al.* 1996a), as many blood vessels terminate near the membrane (Griffin *et al.* 1971). From this site, the bacteria may penetrate the basement membrane and the yolk after invading the granulosa cells or by migrating between the cells and crossing the perivitelline layer. *S.* Enteritidis is able to penetrate the vitelline membrane and multiply within the interior yolk contents after deposition onto the exterior surface of intact egg yolks (Gast and Holt 2001a). *S.* Enteritidis bacteria are also found associated with the yolk membrane after oral inoculation of laying hens (Gast and Holt 2001a). However, in another report most of the ovarian infections after i.v. inoculation with *S.* Enteritidis were found to be confined to the interstitial tissues and not to the yolk contained in the large follicles (Barrow and Lovell 1991).

5. COLONIZATION OF THE OVIDUCT

5.1 Mode of access to the oviduct

In many reports (Timoney *et al.* 1989; Shivaprasad *et al.* 1990; Humphrey *et al.* 1991b,c; Thiagarajan *et al.* 1994; Keller *et al.* 1995) contamination of eggs is associated with isolation of *S.* Enteritidis from the oviduct, suggesting a contamination of the egg in these locations. Miyamoto *et al.* (1997) observed that when eggs are developing in a highly contaminated oviduct, they are likely to be contaminated with the organism. *Salmonella* bacteria have been found on the mucosal surface and within epithelial cells, lining the oviduct in naturally infected hens (Hoop and Pospischil 1993). This was previously interpreted as the sole result of an ascending infection from the cloaca. Several studies indeed have focused on ascending infections and the role of

the vagina in the production of *S*. Enteritidis-contaminated eggs (Barrow and Lovell 1991; Keller *et al.* 1995; Reiber *et al.* 1995; Reiber and Conner 1995; Miyamoto *et al.* 1997, 1999). Intravaginal infection tends to ascend only to the lower parts of the oviduct (Miyamoto *et al.* 1997), but can cause the contamination of forming eggs (Miyamoto *et al.* 1999). The subsequent contamination of the egg following intravaginal infection takes place in the isthmus, uterus, vagina or cloaca and rarely in the upper oviduct and ovaries (Okamura *et al.* 2001b).

It is now widely accepted that ascending infections from the cloaca is not the only mode of access of the bacteria to the oviduct. Prior to eggshell deposition, forming eggs can be subject to descending infections from colonized ovarian tissue, ascending infections from colonized vaginal and cloacal tissues, and lateral infections from colonized upper oviduct tissues (Keller *et al.* 1995). Translocation of organisms from the peritoneum to the oviduct via macrophages also has been suggested (Snoeyenbos *et al.* 1969; Turnbull and Snoeyenbos 1974; Turnbull and Richmond 1977).

Once present in the lumen of the oviduct, *Salmonella* bacteria can contaminate forming eggs and establish a long-lasting colonization of the oviduct tissue. Numerous experimental studies have been carried out to elucidate the pathogenesis of oviduct colonization. Colonization of the oviduct with *S*. Enteritidis has been recorded after experimental oral infection (Barrow and Lovell 1991; Kinde *et al.* 2000; Gast *et al.* 2002), intravenous infection (Barrow and Lovell 1991; Kinde *et al.* 2000; Gast *et al.* 2002), intravaginal and intracloacal infection (Miyamoto *et al.* 1997) and inoculation by aerosol (Baskerville *et al.* 1992; Gast *et al.* 2002). In all cases the infection resulted in contamination of eggs. However there is some dispute about the influence of the challenge route on the frequency of egg contamination. Parenteral and aerosol administration lead to higher egg contamination by *S*. Enteritidis (Petter 1993; Miyamoto *et al.* 1997; Henzler *et al.* 1998; Leach *et al.* 1999; Lu *et al.* 1999) and higher reproductive organ infection (Kinde *et al.* 2000) than the oral inoculation in most studies, while this is contradicted in one other study (Gast *et al.* 2002). Intravenous infection resulted in half the developing eggs becoming *Salmonella* positive, in contrast to less contamination using other infection routes (Miyamoto *et al.* 1997). However, the total production of eggs decreases dramatically when laying hens are infected with high intravenous doses of *S*. Enteritidis (Gast *et al.* 2002). Some reports indicate a decrease in egg production after oral inoculation (Gast and Beard 1990b; Shivaprasad *et al.* 1990), while other conclude that there is no adverse effect on egg production (Bichler *et al.* 1996).

Inoculation of oviduct loops has shown that *S*. Enteritidis can colonize the tubular glands of the oviduct (De Buck *et al.* 2004a). Bacteria can be found intracellularly in these glands.

Some non-conventional inoculation models have been shown to result in contamination of eggs. Contamination by *S*. Enteritidis was achieved by using semen as a vehicle for transmission of salmonellae to the hen (Reiber *et al.* 1995). Intramuscular infection with *S*. Enteritidis can lead to the production of internally contaminated eggs (Nakamura *et al.* 1993). Administration of *S*. Enteritidis on to the conjunctivae of laying hens resulted in systemic infection and colonization of the ovary and oviduct, but limited egg contamination (Humphrey *et al.* 1992). Finally, intraperitoneal infections of Japanese quail also resulted in contamination of eggs (Takata *et al.* 2003).

5.2 Bacteria/host cell interactions in the oviduct

Little is known about the colonization mechanism of *Salmonella* in the oviduct of the laying hen. An adaptation of *S*. Enteritidis to the reproductive organs is believed to occur, as a series of *in vivo* passages and subsequent isolation of the bacteria from the reproductive organs, resulted in higher efficacy of egg contamination (Gast *et al.* 2003). Several independent studies have investigated the role of type 1 fimbriae in the interaction of *S*. Enteritidis with the hen's oviduct. *Salmonella* Enteritidis adheres to the surface of the epithelium of the chicken oviduct by type 1 fimbriae (Li *et al.* 2003). Type 1 fimbriae have also been shown to bind the secretions of the isthmus glands, which constitute the eggshell membranes (De Buck *et al.* 2003). It was hypothesized that the binding of *S*. Enteritidis to isthmal secretions could play a role in the contamination of eggs through incorporation of the bacteria in the shell membranes (De Buck *et al.* 2003). Intravenous infection of laying hens with a type 1 fimbriae knock-out mutant resulted in prolonged bacteraemia, a higher frequency of reproductive tract infection but reduction of egg contamination as compared with the parent *S*. Enteritidis strain (De Buck *et al.* 2004b). This is in contrast with a previous study, where no difference in the isolation of *S*. Enteritidis from the reproductive organs and egg contents was found after oral inoculation with three wild type *S*. Enteritidis strains with differential fimbrial expression *in vitro* (Thiagarajan *et al.* 1996b).

Colonization of the oviduct is not limited to adherence to the surface epithelium and to the secretions. Indeed, association of serovar Enteritidis with tubular gland cells of the oviduct has been observed after natural (Hoop and Pospischil 1993) and after experimental infection (Keller *et al.* 1995). Recently, the tropism of *S*. Enteritidis for the tubular gland cells has been shown in an intravenous infection model (De Buck *et al.* 2004a). In addition, the capacity of *S*. Enteritidis to invade and proliferate in these cells has been demonstrated *in vitro*. Inoculation of *S*. Enteritidis directly into the lumen of the oviduct, in an

in vivo oviduct loop model, again resulted in invasion of the tubular glands (De Buck *et al.* 2004a). Invasion not only implicates colonization of gland lumina but also presence of intracellular bacteria. *Salmonella*-infected host cells are distributed over the length of the oviduct (Hoop and Pospischil 1993). *S.* Enteritidis has been identified by immunohistochemistry not only on the mucosal surface and inside the mucosal epithelium but also deeper in the stromal tissues of the oviduct of Japanese quail after intraperitoneal inoculation. Many of the bacteria are contained in the cytoplasm of mucosal epithelial cells and stromal cells in these tissues (Takata *et al.* 2003).

Different segments of the oviduct may differ in their susceptibility to *S.* Enteritidis colonization and invasion. Higher numbers of intracellular *S.* Enteritidis bacteria were found in the isthmus than in the magnum, both in an intravenous infection model and in inoculated oviduct loops (De Buck *et al.* 2004a). In vitro, the invasion of *S.* Enteritidis is higher in cultured tubular epithelial cells of the isthmus than of the magnum (De Buck *et al.* 2004a). These observations are in accordance with the results of most experimental infections (Keller *et al.* 1995; Bichler *et al.* 1996; Okamura *et al.* 2001b), where the isthmus is the most frequently and heavily contaminated segment of the oviduct. Analysis of surface decontaminated eggs laid by infected hens has shown that the shell, containing the eggshell membranes, produced by the isthmus, is often the most heavily infected site (Bichler *et al.* 1996; Miyamoto *et al.* 1997; Okamura *et al.* 2001b). From the results of bacterial culturing of oviduct segments and eggs after experimental infections it is concluded that *S.* Enteritidis has adapted best to the isthmus segment of the chicken oviduct.

Hens lay contaminated eggs in a clustered and intermittent way (Humphrey 1989), possibly caused by the occasional reappearance of bacteria from the infected tissue into the lumen of the oviduct (Keller *et al.* 1995). This egression might be induced by stress, hormonal variations, fluctuations in the immunological protection or other unknown factors. Similarly, the stress during molting has been demonstrated to cause more shedding of *S.* Enteritidis in the faeces (Holt and Porter 1992a,b; Holt *et al.* 1994, 1995; Holt 2003).

6. WHY IS *S.* ENTERITIDIS THE PREDOMINANT SEROTYPE FOUND IN EGGS?

Salmonella Enteritidis is not unique in invading the intestinal tissue of the chicken, nor is it unique in causing bacteraemia and spreading to the internal organs, or in colonizing the ovaries (Barnhart *et al.* 1993; Okamura *et al.* 2001a) or oviduct (Keller *et al.* 1997). Nevertheless, *S.* Enteritidis is the predominant serotype found in eggs, while only sporadically other *S.* serovars, i.e. mostly *S.* Typhimurium, can be isolated from eggs (Chapman 1988; Indar *et al.* 1998; Williams *et al.* 1998; Leach *et al.* 1999; Sarna *et al.* 2002; Tribe *et al.* 2002). Moreover, as mentioned above, *S.* Enteritidis is by far the most common serotype associated with egg-borne salmonellosis in humans and *S.* Enteritidis is also by far the most common serotype isolated from laying hens.

At the present time, it is unclear why *S.* Enteritidis is the predominant serotype associated with laying hens and with eggs, although a number of hypotheses can be formulated. One possible explanation is that *S.* Enteritidis might have a higher tropism and affinity for the reproductive organs of the hen than other serovars. Of six serovars, *S.* Enteritidis and Typhimurium are the only serovars that are able to colonize the reproductive organs (Okamura *et al.* 2001a). Enhanced tropism for the reproductive tract has been reported for other *Salmonella* serotypes in other hosts: *S.* Abortusequi and *S.* Abortusovis also have the ability to colonize the reproductive organs, in horses and sheep respectively (Padron *et al.* 1988; Madic *et al.* 1997). The association between reproductive tract colonization and egg contamination is also found in *S.* Gallinarum biovar Pullorum (Gast 1997; Wigley *et al.* 2001).

Isolates or serovars of *Salmonella* that are less aggressive in the reproductive tract might have an advantage over more aggressive ones to end up contaminating eggs. A *S.* Typhimurium challenge causes pathological lesions in layers, including atrophy or shrinkage of the oviduct, and, in some cases, total cessation of egg production. In contrast, *S.* Enteritidis does not cause grossly visible pathological lesions (Hassan and Curtiss 1997). *S.* Enteritidis can be recovered at a low frequency from all internal organs sampled from laying hens for as long as 22 weeks after exposure (Gast and Beard 1990a). Heavily infected follicles in the ovary have been suggested not to develop up to the phase of ovulation because the development of salmonella-infected follicles is prevented by degenerative and inflammatory processes (Matthes and Hanschke 1977). Here again, isolates or serovars that would not cause such processes, would be able to contaminate eggs. This hypothesis was already previously formulated as follows: *S.* Enteritidis is the cause of the food-borne salmonellosis pandemic in humans, in part because it has the unique ability to contaminate eggs without causing discernible illness in the birds infected (Guard-Petter 2001).

Another possible explanation for the preferential egg association of *S.* Enteritidis is that this serovar may have virulence factors that enhance the chances to reach the ovary or oviduct in the course of an infection. Indeed, it has been observed that *S.* Enteritidis persists longer in the blood after intravenous inoculation than other *Salmonella* serovars (Okamura *et al.* 2001a). It is possible that *S.* Enteritidis

can infect eggs more efficiently because of this capacity of prolonging the bacteraemic phase of an infection, or perhaps *S*. Enteritidis can repeatedly cause septicaemia to spread from colonized organs such as the spleen to the reproductive organs, as *S*. Gallinarum biovar Pullorum is suggested to do (Wigley *et al.* 2001). Furthermore, it has been shown that *S*. Enteritidis colonizes the internal organs, especially the ovary, more consistently than do the other serovars after intravenous inoculation (Okamura *et al.* 2001a).

Most likely, *S*. Enteritidis possesses a unique set of virulence factors in relation to egg contamination or has a unique regulation of known virulence mechanisms. Suggestions have been made in this respect about the expression of type 1 fimbriae (De Buck *et al.* 2003, 2004b) or the composition of lipopolysaccharide (LPS) (Guard-Petter *et al.* 1997). The presence of high-mass molecular LPS (HMM LPS) on *S*. Enteritidis was correlated with egg contamination (Guard-Petter 2001). However, it is not known whether this HMM LPS interacts directly with the reproductive tissue or forming eggs. Recently, a gene of *S*. Enteritidis was suggested to play an essential role in the repair of DNA damage caused by egg albumen and hence provide an advantage to *S*. Enteritidis to survive in chicken eggs (Lu *et al.* 2003).

7. IMMUNE RESPONSE IN INFECTED OVARIES AND OVIDUCTS

The contamination rate of egg contents with pathogenic agents in the oviduct is fairly low, although the vagina opens to the cloaca (Shivaprasad *et al.* 1990; Poppe *et al.* 1992). This is possibly due to the local immunity in the oviduct. The colonization of *Salmonella* in the reproductive tract triggers the immune system to counter the infection. First a non-specific response can be seen in most of the ovaries and oviducts of infected laying hens as slight inflammatory processes with heterophil infiltration occur, varying from focal to diffuse in distribution (Hoop and Pospischil 1993).

Almost all T cell subsets in the ovary and different regions of the oviduct increase in number at 7 days post-inoculation and peak at day 10. This T cell surge is followed by a peak in B cell numbers at day 14. The number of macrophages declines initially but recovers to preinoculation levels by day 21. At day 21, the numbers of T and B cells return to normal levels, except for IgG+ B cells in the infundibulum, isthmus, and vagina which remain consistently elevated. The decline in *S*. Enteritidis-positive tissues from infected hens beginning at day 14 is suggested to be associated with the T and B cell proliferation at 10–14 days post-inoculation, indicating a major role of the local immune response to *S*. Enteritidis for these lymphocytes (Withanage *et al.* 1998).

Protection from *S*. Enteritidis infection by humoral mechanisms alone is unlikely, because of its facultative intracellular nature. Despite the rapid production and secretion of *S*. Enteritidis-specific antibodies in infected laying hens, complete clearance of *S*. Enteritidis from the oviducts of infected hens does not occur (Withanage *et al.* 1999). *S*. Enteritidis can be recovered from ovaries and oviducts for as long as 22 weeks after exposure (Gast and Beard 1990a). Hence, persistence in these tissues occurs in spite of activation of the immune response. Similarly, persistence of *S*. Pullorum in the chicken occurs despite high levels of circulating specific antibodies (Wigley *et al.* 2001). A relationship between the *S*. Enteritidis-specific antibodies and the declining bacterial recovery from the reproductive organs has been reported (Withanage *et al.* 1999). Others found no direct relationship between the magnitude of the antibody responses of individual hens and the frequency at which they lay contaminated eggs (Humphrey *et al.* 1991a). Secretion of *Salmonella*-specific antibodies in the oviducts of hens experimentally infected with *S*. Enteritidis has been shown (Withanage *et al.* 1999). IgG, IgM and IgA are known to be released into the oviduct lumen from the infundibulum, magnum, isthmus and uterus (Kimijima *et al.* 1990).

The dynamics of the antibody levels in the oviduct are identical to those in the serum. IgG and IgM levels in oviducts and in sera reach a peak by 14 days post-inoculation, and remain elevated throughout. The secretion of IgA seems to be transient as the IgA levels increase to a peak 7 days after both primary and secondary inoculations, and decline rapidly (Withanage *et al.* 1999).

The magnitude of the antibody responses detected in individual hens may not predict the overall risk of egg contamination associated with particular laying flocks (Gast and Holt 2001b). Birds infected with *S*. Enteritidis produce positive eggs at high frequencies initially but decreasing over time. However, when *S*. Enteritidis antibodies begin to decrease, the frequency of *S*. Enteritidis-positive eggs increases again (Bichler *et al.* 1996).

Older laying hens are more adversely affected by *S*. Enteritidis (acute diarrhoea, prolonged faecal carriage, strong septicaemia) and fail to elaborate significant levels of antibodies (Humphrey *et al.* 1991b). Moulting hens are more susceptible to *S*. Enteritidis infection (Holt 2003). A possible mechanism may be the depression of cellular immunity in moulting hens (Holt 1992b) and the reduction of the number of CD34+ T cells in the peripheral blood (Holt 1992a), induced by moulting.

8. PROTECTION AGAINST REPRODUCTIVE TRACT INFECTION IN LAYING HENS

The association between reproductive tract infection in layers, egg contamination and human food poisoning by *S*. Enteritidis should be a strong incentive to develop control

programmes directly aiming at the reduction of laying hen infections. This can be achieved by reducing the infection pressure in the environment of the hen and by increasing the resistance of the hen against infections.

8.1 Vaccination

The goal of vaccinating laying hens is to reduce or even completely suppress egg contamination. At the present time, two types of *Salmonella* vaccines for laying hens exist: the live vaccines and the bacterins. Both types of vaccines afford a certain level of protection. The first commercial vaccines were inactivated vaccines. Inactivated whole-cell killed *Salmonella* vaccines confer partial protection against intestinal colonization, faecal shedding, systemic spread and egg contamination in chickens. An intravaginal challenge model in hens clearly demonstrated the ability of a *S*. Enteritidis oil-emulsion bacterin to protect against egg contamination (Miyamoto *et al.* 1999). However, in one study, flocks vaccinated with a bacterin gave similar results as unvaccinated flocks in regard to contamination of the organs of the bird (including ovary and oviduct) and the eggs (Davison *et al.* 1999). Although a *Salmonella*-specific humoral response in infected laying hens cannot eliminate the bacteria from the reproductive tract, the presence of immoglobulins in the reproductive tract before the challenge might be sufficient to reduce reproductive tract colonization and the subsequent egg contamination. Moreover, a strong inhibition of the growth of *S*. Enteritidis in egg contents from hens vaccinated with *S*. Enteritidis bacterin occurs compared with non-vaccinated (Holt *et al.* 1996).

The first live *Salmonella* vaccines were spontaneous mutants or strains attenuated empirically by chemical or u.v. mutagenesis. Live attenuated *Salmonella* vaccines are potentially superior to induce cell-mediated immunity in addition to antibody responses. These vaccines can be administered through the drinking water. They induce immune responses at multiple mucosal sites. They can be produced at low cost and they are easy to store. The threat of residual virulence or reversion to virulence is the major disadvantage of the live vaccines.

The recent advances in the understanding of the genetics of *Salmonella* virulence has led to the development of attenuated *Salmonella* strains with single or multiple defined mutations in known virulence genes. Vaccination of laying hens with live avirulent *Δ*cya *Δ*crp *Salmonella* Typhimurium bacteria entirely prevented transmission of *S*. Enteritidis and Typhimurium into eggs, with no effect on egg production (Hassan and Curtiss 1997). However, sporadic isolation of *Salmonella* challenge bacteria from the oviduct of vaccinated hens, indicates that vaccination is not fully protective at the tissue level (Hassan and Curtiss 1997). Vaccination with a *ΔaroA S*. Typhimurium modified live vaccine did not reduce internal egg contamination by *S*. Enteritidis (Parker *et al.* 2001).

All in all, vaccination of laying hens in several studies leads to significantly reduced egg contamination. As such, vaccination undeniably is a useful tool as part of a comprehensive control programme for *Salmonella* infections in layers. Laying hen vaccination has been implemented in national control programmes in several countries. A decline in the number of recorded human cases of *S*. Enteritidis infection in the UK in the last 5 years has been observed concomitant with the introduction of vaccination of egg-laying hens against serovar Enteritidis (Cogan and Humphrey 2003).

8.2 Other measures

Other strategies to control *Salmonella* infection in laying hens aim at preventing intestinal colonization, based on the use of prebiotics, probiotics, synbiotics and other feed additives (Van Immerseel *et al.* 2002). *Lactobacilli* derived from the cloaca and vagina of laying hens are believed to confer a protective effect against *S*. Enteritidis colonization in the cloaca and vagina. An inhibitory activity against *S*. Enteritidis has been demonstrated in an in vitro inhibition assay (Miyamoto *et al.* 2000). The use of those *Lactobacilli* as a probiotic is therefore a promising control measure against *Salmonella*, specifically for laying hens. Intensive research is needed on the administration of such probiotic products to reach the cloaca and vagina as well as on their effectiveness.

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