

ISSN 1682-8356  
ansinet.org/ijps



INTERNATIONAL JOURNAL OF  
**POULTRY SCIENCE**

**ANSI***net*

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## Study on Two Inoculation Routs of *Salmonella enteritidis* in Abilities to Colonize in Internal Organs and to Contaminate of Eggs in Broiler Breeder Hens

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**Abstract:** Two groups of chickens were inoculated orally and intravenously with  $10^{10}$  and  $10^6$  CFU *S. enteritidis* organisms consequently. Heavier infection of liver spleen, caecum, small intestines, infundibulum-ovules and cloac-vagina of chickens that inoculated orally were observed. In intravenously inoculated group high infection of liver-spleen and cloac- vagina were noticed. In oral group egg production were more decreased and fecal shedding was higher than intravenously group.

**Key words:** *S. enteritidis*, oral, intravenous, chicken, egg production

### INTRODUCTION

Salmonellosis is one of the most important food-borne diseases. The World Health Organization (WHO) has reported 1.3 billion cases per year of acute gastroenteritis due to non-typhoid salmonellosis with 3 million fatal cases (Gomez *et al.*, 1997).

In 2006, a total of 165, 023 confirmed cases of human salmonellosis were reported in the European Union. In this report, the prevalence *S. enteritidis* was identified 62.5% and *S. Typhimurium* was 12.9%. The overall European Union prevalence of *Salmonella* in table eggs was 0.8% in 2006 and >90% of all egg isolates were *S. enteritidis* whereas, *S. enteritidis* was the most common serotype (52.3%) in the laying flock environment (EFSA, 2007). The persistence of this organism in poultry house environments poses a continuing threat of infection for laying hens (Davies and Breslin, 2003; Kinde *et al.*, 2004; Lapuz *et al.*, 2008). Additionally, there is suggestion that *S. enteritidis* has some intrinsic characteristics that allow a specific interaction with either the reproductive organs of laying hens or the egg components (Gantois *et al.*, 2009).

In poultry, an important step in salmonella pathogenesis is bacterial entry in the epithelial cells of the intestinal tract, especially the caeca (Desmidt *et al.*, 1996). *Salmonella* actively stimulates its own uptake into epithelial cells by inducing cytoskeleton rearrangements and membrane ruffling (Finlay and Falkow, 1989). These morphological changes are triggered by proteins secreted of *Salmonella* into the cytosol of the epithelial cells via a type III secretion system (TTSS) encoded by genes of the *Salmonella* pathogenicity island 1 (SPI-1) (Mills *et al.*, 1995; Darwin and Miller, 1999). Several regulatory proteins that are involved in *Salmonella* invasion have been characterized (Lucas and Lee,

2000). The key regulator of SPI-1 is *hilA*, a transcriptional activator encoded on SPI-1 that regulates the expression of the SPI-1 secretion system as well as many of its secreted effectors (Bajaj *et al.*, 1995).

Oral infection of hens with *S. enteritidis* has led to the invasion of a variety of internal organs, including the ovary and oviduct (Gast and Beard, 1990) and produced sporadic egg contamination for several weeks (Gast and Holt, 2000; Okamura *et al.*, 2001a).

The colonization of reproductive tissues in infected laying hens is a pivotal stage in the production of contaminated eggs that can transmit *S. enteritidis* infections to offspring (Gast *et al.*, 2009; Okamura *et al.*, 2001a,b).

Egg contamination is caused by penetration through the eggshell by *S. enteritidis* contained in feces after the egg is covered by the shell (Messens *et al.*, 2005; De Reu *et al.*, 2006). The second possible route is by direct contamination of yolk or albumen originating from the infection of reproductive organs with *S. enteritidis* before the egg is covered by the shell (Timoney *et al.*, 1989; Keller *et al.*, 1995; Miyamoto *et al.*, 1997; Okamura *et al.*, 2001a,b). The location of *S. enteritidis* deposition in a developing egg (yolk or albumen) is likely a consequence of which regions of the laying hen's reproductive tract are colonized (Bichler *et al.*, 1996; Gast and Holt, 2000; Humphrey *et al.*, 1991).

Base of these, aim of study trail was to establish a model infection of *S. enteritidis* in laying hens in which the internal organs e.g. digestive or reproductive systems could become infected and consequently the incidence of contaminated eggs could be studied. Therefore, hens were inoculated intravenously and orally. Different tissue samples were taken for salmonella recovery.

## MATERIALS AND METHODS

**Bacterial strain:** *S. enteritidis* phage type 4, strain NIDO 76Sa88 Nalr (parent strain) was used in this experiment, obtained from Ghent University, Belgium. The nalidixic acid resistant strain is well-characterized (Desmidt *et al.*, 1996; Van Immerseel *et al.*, 2002).

**Hens:** Fifty 26-week-old broiler breeder hens were selected from an Arian Grand Parent farm that is under strict control for Salmonella and other infectious diseases. They were free of any apparent disease throughout the growing and laying periods. Hens were divided into two groups. Before starting of the experiment cloacal swabs were taken from all hens and checked for Salmonella infection, to confirm that animals were Salmonella-free.

Hens randomly divided in two groups of 25 birds. First group was inoculated intravenously (IV) with  $10^6$  CFU of *S. enteritidis* 76Sa88 Nalr parent strain bacteria, using 0.1 ml of PBS and second group hens were inoculated by oral (OR) route in the crop, using a plastic tube with  $10^{10}$  Colony Forming Units (CFU) of same bacteria in a volume of 1 ml of PBS, as reported previously (Barrow and Lovell, 1991).

At days 2, 7, 14, 21 and 35 post-inoculation, two hens per group were euthanized and post-mortem examinations were carried out. For bacterial analysis samples were taken from different parts of digestive (caecal, small intestine, liver- spleen) and reproduction (infundibulum-ovules, magnum, isthmus, cloaca-vagina) systems separately. Cloacal swabs were taken on same days and examined for *S. enteritidis*. Every 10 eggs were pooled and cultured.

**Bacteriological analysis:** Swabs from cloacae were placed in 5 ml selenite cysteine broth and after 24 h incubation in 37°C, were cultured on Salmonella-Shigella (SS) agar plates. Suspected colonies were cultured in Triple Sugar Iron Agar (TSI) and urea broth tubes. Samples of internal organs were homogenized and 10-fold dilutions made in PBS. For each dilution 100 µl inoculated on SS agar plates with 20 µg/ml nalidixic acid. After overnight incubation (37°C) the number of CFU/g tissue was determined by counting the bacterial colonies (Bohez *et al.*, 2006). For samples which were negative after titration, pre-enrichment and enrichment was performed in selenite cysteine broth. Samples that were negative after titration but positive after Salmonella enrichment were presumed to contain  $10^1$  CFU/g organs. Samples that were negative after enrichment were presumed to have 0 CFU/g. The mean CFU/g tissue was calculated for each group.

On experimental daily basis, every 10 eggs were pooled together into sterile honey jars and contents mixed and homogenized by shaking the jars. These were incubated at 37°C for 48 h and then plated onto the antibiotic containing SS agars.

## RESULTS

Following of inoculation, productivity decreased to a low level and that was more pronounced in oral group (Table 1). Whether this was a result of *S. enteritidis* infection or simply from handling and inoculation is unclear.

Table 1: Egg production after infection of hens by orally (OR) or intravenously (IV) with *Salmonella enteritidis*

Weekly post inoculation	OR	IV
1	43/25*	52/25
2	49/23	63/23
3	43/21	64/21
4	43/19	60/19
5	49/17	57/17

\*Daily percentage of egg production/number of hens



Pic. 1: Inspissated ovary and misshapen follicles in a bird that autopsied in second weeks post infection

In oral group, at the second and third weeks, birds that necropsied had some inspissated ovary and misshapen follicles. *S. enteritidis* was isolated from these organs and from the small intestines, caecum, and oviduct (Picture 1).

Figure 1 summarizes the detection of *S. enteritidis* by bacterial isolation from the different parts of digestive system. *S. enteritidis* was isolated from different parts of gastrointestinal throughout the sampling times, but the majority of detection was from the hens which inoculated orally compared to the IV group birds. In the oral group, the highest recovery rates of *S. enteritidis* were made from caecum at 2, 7 and 14 dpi, while in the group of IV infectious hens, the highest *S. enteritidis* recovery observed from the liver-spleen tissues at 2 and 7 dpi (Fig. 1).

However, the total recovery of *S. enteritidis* from different parts of reproduction system was lower than to the digestive system but the majority of isolates from this system were obtained in IV group compared than to those of OR group hens (Table 2). In the reproduction system, the highest recovery of *S. enteritidis* was

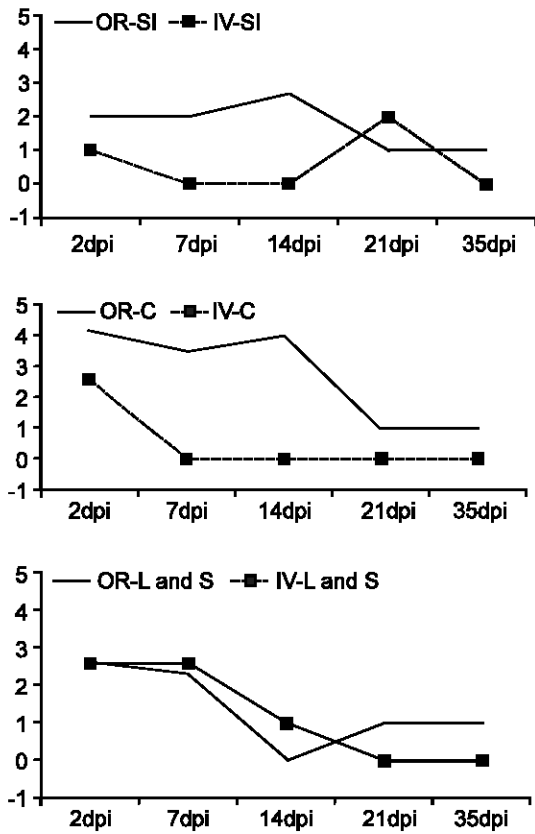


Fig. 1: Comparison of salmonella counts (log<sub>10</sub> CFU/g) in different parts of digestive system in two groups of hens that inoculated orally (OR) or intravenously (IV) inoculated with *S. enteritidis*. OR = oral IV: Intravenous SI = Small intestine C = Caecum L and S = Liver and Spleen dpi = days post infection

performed from the cloac-vagina and thereafter infundibulum-ovules tissues.

Egg pool cultures were positive at 7, 14 and 21 dpi in oral group whereas it was positive at 2, 14 and 35 dpi for IV inoculated hens (Table 2). Cloacal swabs were 64% positive at 2dpi and decreased to 23.5% at 35 dpi in oral group, as there was 56% positive at 2 dpi in intravenous inoculated birds but decreased to 17.6% at 35 dpi (Table 2).

**DISCUSSION**

Natural infection of poultry by Salmonella occurs via oral route and salmonella colonize the intestinal tract with the crop and ceca being the primary sites of colonization (Brownwell *et al.*, 1970; Soerjadi *et al.*, 1981; Stavric, 1987; Impey and Mead, 1989). In the present study, contamination of gastrointestinal organs in OR group was higher than to IV group. Additionally, recovery of *S. enteritidis* from caecum of oral group was higher than IV group. The ceca have been recognized as the region for the most frequent recovery of Salmonella after oral infection. Okamura (2001b) explained that after IV inoculation, *S. enteritidis* could keep bacteremia and remained persistently in the liver and ceca to a high degree. In this study caecum was infected with *S. enteritidis* when hens inoculated via intravenously too. It could be considered that the ceca may contaminate from the liver by the gallbladder secretion.

As observed, *S. enteritidis* recovery from infundibulum-ovules and cloac-vagina were more appeared in comparison with magnum and isthmus. On the other hand, the colonization of *S. enteritidis* in ovary and preovulatory follicles of IV group were clearly higher than oral group that confirmed the previous reports (De Buck *et al.*, 2004; Gantois *et al.*, 2006). In the majority of these studies in laying hens, a higher frequency of ovary colonization is reported, compared with the frequency of recovery from other sections of the oviduct (De Buck *et al.*, 2004; Gast *et al.*, 2007). Because, it is strongly believed that *S. enteritidis* must interact with the cellular components of the preovulatory follicles. The extensive permeability of the vascular endothelia observed in the ovary may contribute to the high colonization rate at this site (Griffin *et al.*, 1984). Oviduct infection in IV group appeared to be the result of haematogenous spread (Barrow and Lovell, 1991) and in oral group it is generally believed that colonization of the reproductive organs is a consequence of systemic spread of Salmonella from the intestine (Vazquez-Torres *et al.*, 1999).

Eggs contents pool culture results were not consistent at different days but it seems at a time that salmonella were isolated from infundibulum-ovule or cloac-vagina, these cultures would be positive.

Table 2: Recovery and counts (log<sub>10</sub> CFU/g) of *Salmonella enteritidis* from different parts of reproduction system and the cloacal swaps in two group hens that inoculated orally (OR) and intravenously (IV) with *Salmonella enteritidis*

Reproduction system	2 dpi		7 dpi		14 dpi		21dpi		35 dpi	
	OR	IV	OR	IV	OR	IV	OR	IV	OR	IV
Infundibulum-ovules	0	0	0	1	1	1	1	1	0	1
Magnum	0	0	0	0	0	0	0	1	0	0
Isthmus	0	0	0	0	0	0	0	0	0	0
Cloac-vagina	2	2	0	1	1	0	1	1	0	1
Eggs contents culture	-	+	+	-	+	+	+	-	-	+
Cloacal swaps	64%	56%	26%	52%	33%	29%	37%	26%	24%	18%

Numerous studies have also been performed to investigate the effect of the inoculation route on the production of contaminated eggs (Miyamoto *et al.*, 1997; Gast *et al.*, 2002). While Gast *et al.* (2002) reported that oral, aerosol and intravenous inoculations led to similar frequencies of egg contamination. Miyamoto *et al.* (1997) observed a higher contamination rate when birds were inoculated intravenously and intravaginally. Our data indicated that most parts of digestive system infected when birds inoculated orally, as may observe in the natural conditions in the field. Whereas, the majority of *S. enteritidis* recovered from reproductive system was in intravenously group. This indicates that the main route of contamination of reproductive system might be through the systemic infectious, as was reported previously. However, under the various conditions and routes by which chickens might become infected by *S. enteritidis* phage type 4, eggs are more likely to become contaminated during passage through the cloaca and/or as a result of ovarian infection.

#### ACKNOWLEDGMENT

This research was funded by the research committee, University of Tehran, Faculty of Veterinary Medicine.

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