

Inoculation of a Poultry Isolate *Salmonella enteritidis* on Egg Vitelline Membrane: Survival and Growth in Egg Components after Different Refrigeration Storage Times

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Abstract: An *in vitro* study was designed to determine the extent of *Salmonella enterica* serovar enteritidis survival and growth permissiveness in egg components isolated from shell eggs held at refrigeration temperature over an 8 week time period. Eggs were collected from a commercial laying facility at one-week intervals for eight weeks and stored at refrigeration temperature. After storage, eggs were dipped in ethanol, cracked aseptically and separated into yolk and albumen samples. *S. enteritidis* resistant to novobiocin and nalidixic acid were inoculated on to the surface of the yolk membrane at a concentration of approximately 10^6 CFU mL⁻¹. Yolks were then covered with albumen and incubated for 24 hrs at 25°C. After incubation, eggs were separated into component parts. Samples were removed from yolk, albumen and yolk membrane and diluted 10-fold in sterile phosphate buffered saline. In albumen, *S. enteritidis* counts were increased in weeks 3 and 8 compared to week 1 (trial 2). The frequency of eggs exhibiting net growth of *S. enteritidis* in albumen occurred at week 7 versus weeks 0 and 1 in trial 1 and weeks 3 and 8 versus weeks 0 and 2 in trial 2. In the membrane fraction, the frequency of eggs exhibiting net growth of *S. enteritidis* occurred at weeks 5 and 8 versus week 0 in trial 2. In the yolk fractions, *S. enteritidis* counts recovered from week 6 eggs were significantly higher ($P < 0.05$) than those of weeks 0, 2, 3 and 7 (trial 2) and the number of detectable *S. enteritidis* positive eggs were greater in week 8 than week 5 in trial 1. This suggests that egg components recovered from aged eggs stored at refrigeration temperatures infrequently supported *S. enteritidis* net growth but generally did not inhibit survivability.

Keywords:

INTRODUCTION

Salmonella enterica serovar Enteritidis (*S. enteritidis*) accounts for over 65% of all human serotype isolates worldwide^[1]. Beginning in the late 1970's infections of this pathogen spread over the next ten years from New England to the remainder of the United States^[2, 3]. Although broiler chicken carcasses have shown recent increases in this serotype, Grade A shell eggs are usually considered the most common

vehicle for the transmission of *S. enteritidis*^[3-5]. Management practices such as feed withdrawal molt induction in laying hens to initiate multiple egg laying cycles have been identified as causes for increased *S. enteritidis* colonization in the gastrointestinal tract^[6-8]. Development of alternative molt diet regimes that avoid feed withdrawal have been shown to limit *S. enteritidis* colonization and infection in susceptible laying hens^[9-16]. However, despite the availability of these management options and introduction of egg quality

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assurance programs, the numbers and incidence continues to persist in some parts of the United States^[2]. This continued trend in disease incidence requires understanding the environmental conditions which favor *S. enteritidis* establishment and persistence in eggs and egg components.

Albumen or egg white possesses several chemical defenses that limit growth of pathogens including lysozyme, ovotransferrin, avidin and ovomucoid^[17]. Ovotransferrin and iron availability are believed to play a major role in limiting *S. enteritidis*^[18]. Although the egg albumen is relatively hostile to *S. enteritidis* multiplication there is evidence that *S. enteritidis* is capable of long term survival in the internal egg environment. In a study by Lock and Board^[19] they suggested that salmonellae introduced onto the air cell of an egg could survive for up to 17 days at refrigeration temperatures. Rizk *et al.*^[20] observed that at high relative humidity salmonellae may be able to survive refrigeration temperature. As length of storage time of shell eggs increases, albumen quality and vitelline membrane strength deteriorate^[21,22]. The breakdown of the vitelline membrane could either allow bacteria such as *S. enteritidis* to gain access to yolk material, or allow nutrient compounds such as iron to leak out into the albumen^[23].

Previously we observed that serotype *S. typhimurium* could survive and exhibit net growth on egg components during an 8 week storage period under refrigeration conditions^[24]. This combination of increased susceptibility for egg deterioration at advanced storage times with the ability of *S. typhimurium* to survive refrigeration makes understanding the time line of an egg's *S. enteritidis* survival and growth permissiveness critical. We hypothesize that an increase in the refrigeration time could increase the susceptibility to *S. enteritidis* contamination. The objective of this *in vitro* study was to use similar vitelline membrane inoculation approaches as our previous work with *S. typhimurium*^[24] and determine whether sufficient deterioration occurs in refrigerated eggs over an 8 week time period such that individual egg components will permit survival and net growth of *S. enteritidis* after inoculation on the vitelline membrane.

MATERIALS AND METHODS

Egg collection: Over the course of eight weeks, eggs were collected from a commercial flock of 60 week old single comb white leghorn hens at one week intervals. Eggs were stored at refrigeration temperatures of 4°C under a relative humidity of 97% for a period of zero to

eight weeks. After refrigerated storage, eggs were separated into time points of 0, 1,2,3,4,5,6,7 and 8 week old eggs and inoculated with *S. enteritidis* prepared as described in the following sections.

Bacterial preparation: A strain of *S. enteritidis* isolated from poultry at the University of Iowa, (Ames, Iowa), resistant to novobiocin and nalidixic acid and known to be infective in laying hens^[10,13-16] was grown overnight in Difco™ Tryptic Soy Broth (Difco Laboratories, Detroit, MI, USA). After incubation, cells were centrifuged for 10 min at 8000 rpm. Supernatant was discarded and cells were resuspended in 6 mL of sterile phosphate buffered saline. The centrifuge and resuspension steps were subsequently repeated. The final concentration of cells was 10⁶ CFU mL⁻¹.

Sampling method: Eggs were dipped in 95% ethanol in order to remove surface contamination of the shell^[25]. Eggs were then aseptically cracked and separated into yolk and albumen components in sterile vessels. A 0.01 mL aliquot of *S. enteritidis* prepared as stated previously was then placed directly on the exposed vitelline membrane of the eggs to be sampled. After holding the inoculated yolk for 5 min to allow bacterial attachment, albumen was added back to the sterile vessel. Ten eggs were inoculated at each time point. Inoculated eggs were incubated at 25°C for 24 hrs. In addition, one egg was sacrificed following the 5 min holding period in order to determine initial population methods of bacteria that survived transfer into egg components. To determine that eggs used were initially free of *Salmonella*, one egg was sampled prior to inoculation with bacteria. Two separate trials were run using this method and the *S. enteritidis* poultry isolate. Both trials used the same set of saved eggs. For each trial 10 eggs were inoculated and later sampled for each time point from 0 to 8 weeks of storage.

After 24 hrs of incubation eggs for all time points including the zero time, stored eggs were again separated into component parts of yolk and albumen. A 1 mL sample of albumen was then drawn using a 3 mL syringe. The following sample method previously described by Gast and Holt^[26] was used to gather yolk samples. A flame heated spatula was applied to the vitelline membrane. This created a sterile opening through which a 16-gauge needle could pass^[26]. Membrane samples were taken by gathering the membrane with flamed rat tooth forceps followed by stomaching with 5 mL of sterile phosphate buffered samples.

All samples were subsequently diluted ten fold in sterile phosphate buffered saline at neutral pH and

plated on Brilliant Green Agar (BGA, Difco Laboratories, Detroit, MI, USA) with novobiocin and nalidixic acid ($25\mu\text{g mL}^{-1}$). Plates were inoculated with 0.01 mL of each dilution and stored for 48 hrs at 37°C. Colonies of typical *Salmonella* morphology were enumerated on BGA.

Statistical analysis: Each trial was designed to consist of samples taken from ten eggs per week. However, because of the fragile nature of the egg components it was not always possible to recover intact egg components. Therefore most data statistically analyzed in this study were based on less than ten eggs and reported accordingly. Population counts from multiple plates of the same dilution series were averaged to give total colony counts from each individual egg. *S. enteritidis* counts were subjected to one-way analysis of variance using GLM procedure of SAS (SAS Institute, 2001). The number of eggs containing detectable *S. enteritidis* and the number of eggs containing higher *S. enteritidis* than the initial inoculation level were analyzed using Chi square analysis. Significant differences among the refrigeration storage times were reported at the 0.05 level.

RESULTS AND DISCUSSION

Albumen: Albumen *S. enteritidis* counts over time, number of eggs containing detectable *S. enteritidis* and number of eggs containing higher *S. enteritidis* than the initial inoculation level are shown in Table 1. *S. enteritidis* was detected in all eggs from all weeks in both trials. In trial 1 there were no significant differences ($P>0.05$) in *S. enteritidis* counts among the eight weeks. However, frequency of *S. enteritidis* positive eggs above the original inoculation number (10^6 colony forming unit) at week 7 was significantly higher ($P<0.05$) than eggs from weeks 0 and 1. In trial 2, *S. enteritidis* counts of weeks 3 and 8 were significantly higher than eggs from week 1. In trial 2, frequency of *S. enteritidis* positive eggs above the original inoculation number (10^6 colony forming unit) at week 3 was significantly higher ($P<0.05$) than weeks 0 and 2. Frequency of *S. enteritidis* positive eggs above the original inoculation number at week 8 was significantly higher ($P<0.01$) than week 0 and 2.

We also observed net growth of *S. typhimurium* in albumen in some eggs at different weeks^[24]. Gast and Holt^[26-28] showed that fresh albumen is inhospitable to the growth of *S. enteritidis*. Hara-Kudo *et al.*^[29] reported that *S. enteritidis* grew less frequently in eggs stored at 10 °C than eggs stored at either 20 or 30 °C and *S. enteritidis* grew more preferably in albumen of

cracked eggs versus intact eggs. Fleischman *et al.*^[30] did not observe *in vitro* growth of *S. enteritidis* inoculated in albumen incubated over 2 days at 4 and 8 °C. It is noteworthy that using the same amount of bacterial cells in the current study, both fresh eggs and those from different storage times were relatively resistant to *S. enteritidis* multiplication in the albumen. However, as indicated by the 100% detection frequency observed in the current study, *S. enteritidis* was still capable of survival in the albumen regardless of the age of the eggs from which the albumen was recovered. Murase *et al.*^[31] also observed survival of *S. enteritidis* after inoculation into separated albumen from eggs stored at 4 °C for 1 to 4 weeks. This survivability would seem to reinforce the idea that if the albumen deteriorates over time, the potential exists for *S. enteritidis* to multiply in the albumen.

Vitelline membrane: Membrane *S. enteritidis* counts over time, number of eggs containing detectable *S. enteritidis* and number of eggs containing higher *S. enteritidis* than the initial inoculation level are shown in Table 2. *S. enteritidis* was detectable in all eggs for all weeks in both trials. Vitelline membrane counts from both trials showed that there were no significant differences ($P>0.05$) in enumerated *S. enteritidis* populations recovered from eggs stored over time. Likewise no significant increases ($P>0.05$) in frequencies of eggs exhibiting net growth of *S. enteritidis* were observed in trial 1. The lack of significance in this sample set could be due to the initial dilution of vitelline membrane sample with 5 mL of sterile phosphate buffered saline. In addition, it was not possible to remove all yolk material from the membrane prior to sampling; this may have led to a variation in sample volume that could have affected variability in the counts taken. Fleischman *et al.*^[30] did not observe *in vitro* growth of *S. enteritidis* inoculated on vitelline membrane incubated over 2 days at 4 and 8 °C.

In trial 2 frequencies of *S. enteritidis* positive eggs above the original inoculation level (10^6 colony forming unit) at weeks 5 and 8 were higher than from eggs at week 0 ($P<0.05$). We also observed net growth of *S. typhimurium* in the vitelline membrane in eggs from different weeks of refrigerated storage^[24]. Immediate refrigeration at 7 °C followed by 24 hrs of refrigerated storage at this temperature has been shown to prevent the *in vitro* penetration of *S. enteritidis* through the yolk membrane^[32]. However, studies by Kirunda and McKee^[22] have demonstrated that with increased storage time, the strength of the vitelline membrane is significantly decreased. Consequently, changes in membrane strength and composition as a

Table 1: Albumen *S. enteritidis* (SE) counts over time, number of eggs containing detectable *S. enteritidis* and number of eggs containing higher *S. enteritidis* than initial inoculation

Inoculation time (Week)	Trial 1			Trial 2		
	Log cfu/mL	Detectable SE in eggs/total (%)	SE net growth in eggs/total (%)	Log cfu/mL	Detectable SE in eggs/total (%)	SE net growth in eggs/total (%)
0	4.84 ± 0.23	8/8 (100)	0/8 (0)	4.62 ± 0.16 ^{ab}	8/8 (100)	0/8 (0)
1	4.56 ± 0.19	8/8 (100)	0/8 (0)	4.39 ± 0.63 ^b	9/9 (100)	2/9 (22)
2	5.00 ± 0.65	5/5 (100)	1/5 (20)	4.69 ± 0.29 ^{ab}	8/8 (100)	0/8 (0)
3	4.92 ± 0.34	4/4 (100)	0/4 (0)	5.87 ± 0.57 ^a	7/7(100)	4/7 (57)**
4	5.13 ± 0.61	10/10 (100)	3/10 (30)	4.71 ± 0.52 ^{ab}	8/8(100)	1/8 (13)
5	5.27 ± 0.45	9/9 (100)	3/9 (33)	4.94 ± 0.23 ^{ab}	7/7(100)	0/7 (0)
6	4.95 ± 0.66	6/6 (100)	2/6 (33)	5.19 ± 0.30 ^{ab}	5/5(100)	1/5 (20)
7	5.91 ± 0.82	7/7 (100)	3/7 (43)*	4.64 ± 0.42 ^{ab}	9/9(100)	1/9 (11)
8	4.83 ± 0.26	7/7 (100)	1/7 (14)	6.05 ± 0.37 ^a	8/8(100)	5/8 (63)***

^{a-b}Means within a column with different superscripts differ significantly (P < 0.05).

* Significant difference compared to weeks 0 and 1 (P < 0.05)

** Significant difference compared to weeks 0 and 2(P < 0.05)

*** Significant difference compared to weeks 0 and 2(P < 0.01)

Table 2: Membrane *S. enteritidis* (SE) counts over time, number of eggs containing detectable *S. enteritidis* and number of eggs containing higher *S. enteritidis* than initial inoculation level

Inoculation time (Week)	Trial 1			Trial 2		
	Log cfu/mL	Detectable SE in eggs/total (%)	SE net growth in eggs/total (%)	Log cfu/mL	Detectable SE in eggs/total (%)	SE net growth in eggs/total (%)
0	5.28 ± 0.21	9/9 (100)	0/9 (0)	5.40 ± 0.20	9/9 (100)	0/9 (0)
1	5.20 ± 0.12	9/9 (100)	0/9 (0)	5.16 ± 0.25	9/9 (100)	1/9 (11)
2	5.03 ± 0.37	7/7 (100)	2/7 (29)	5.02 ± 0.34	8/8 (100)	1/8 (12)
3	4.58 ± 0.21	9/9 (100)	0/9 (0)	5.33 ± 0.26	10/10(100)	2/10 (20)
4	4.98 ± 0.20	10/10 (100)	0/10 (0)	5.48 ± 0.23	9/9(100)	2/9 (22)
5	5.23 ± 0.27	9/9 (100)	2/9 (22)	5.86 ± 0.39	6/6(100)	3/6 (50)*
6	4.90 ± 0.34	6/6 (100)	0/6 (0)	5.42 ± 0.28	5/5(100)	0/5 (0)
7	5.19 ± 0.29	8/8 (100)	2/8 (25)	5.32 ± 0.21	9/9(100)	1/9 (11)
8	5.00 ± 0.31	8/8 (100)	1/8 (12)	5.56 ± 0.30	8/8(100)	4/8 (50)*

* Significant difference compared to week 0 (P < 0.05)

Table 3: Yolk *S. enteritidis* (SE) counts over time, number of eggs containing detectable *S. enteritidis* and number of eggs containing higher *S. enteritidis* than initial inoculation level

Inoculation time (Week)	Trial 1			Trial 2		
	Log cfu/mL	Detectable SE in eggs/total (%)	SE net growth in eggs/total (%)	Log cfu/mL	Detectable SE in eggs/total (%)	SE net growth in eggs/total (%)
0	0.53 ± 0.31	3/9 (33)	0/9 (0)	0.00 ± 0.00 ^b	0/8 (0)	0/8 (0)
1	0.00 ± 0.00	0/8 (0)	0/8 (0)	0.48 ± 0.37 ^{ab}	2/9 (22)	0/9 (0)
2	0.14 ± 0.14	1/7 (14)	0/7 (0)	0.17 ± 0.17 ^b	1/9 (11)	0/9 (0)
3	0.82 ± 0.43	1/9 (11)	0/9 (0)	0.12 ± 0.12 ^b	1/8(12)	0/9 (0)
4	0.59 ± 0.59	1/9 (11)	0/9 (0)	0.32 ± 0.21 ^{ab}	2/8(25)	0/8 (0)
5	0.00 ± 0.00	0/9 (0)	0/9 (0)	0.25 ± 0.25 ^{ab}	1/7(14)	0/7 (0)
6	0.33 ± 0.33	0/6 (0)	0/6 (0)	1.09 ± 0.86 ^a	2/5(40)	0/5 (0)
7	0.00 ± 0.00	0/8 (0)	0/8 (0)	0.10 ± 0.10 ^b	1/10(10)	0/10 (0)
8	1.06 ± 0.53	3/8 (38)*	0/8 (0)	0.27 ± 0.27 ^{ab}	1/8(12)	0/8 (0)

^{a-b}Means within a column with different superscripts differ significantly (P < 0.05).

* Significant difference compared to week 5 (P < 0.05)

function of age may occur to allow multiplication of *S. enteritidis* directly inoculated on the vitelline membrane.

Yolk: Yolk *S. enteritidis* counts over time, number of eggs containing detectable *S. enteritidis* and number of

eggs containing higher *S. enteritidis* than the initial inoculation level are presented in Table 3. In trial 1, frequency of detectable *S. enteritidis* positive eggs at week 8 was significantly higher than eggs from week 5 (P<0.05) whereas there were no significant differences (P>0.05) in *S. enteritidis* counts and frequency of *S.*

enteritidis positive eggs among the treatments. In trial 2, *S. enteritidis* recovered populations from week 6 were significantly higher than those of weeks 0, 2, 3 and 7. However, there were no significant differences ($P>0.05$) in frequencies of detectable *S. enteritidis* positive eggs or *S. enteritidis* positive eggs above the initial inoculation level. Counts taken from yolk samples in both trials were somewhat erratic over time.

Although there were significant peaks in the levels of enumerated *S. enteritidis* populations at different lengths of storage time, there were no noticeable trends in contamination levels over time. Similar trends were also observed for *S. typhimurium* in egg yolks at different weeks^[24]. Weekly time points which yielded substantial differences in the current study were usually the result of only a subset of eggs within the group that supported this net growth. This is probably a reflection of the relatively few egg components in this study that permitted net growth of *S. enteritidis*. Based on *in vitro* studies Gast *et al.*^[33] reported that *S. enteritidis* and *S. heidelberg* could penetrate the vitelline membranes of contaminated eggs in egg yolks during the first day of storage at warm temperatures. Fleischman *et al.*^[30] did not observe *in vitro* growth of *S. enteritidis* inoculated egg yolk incubated over 2 days at 4 and 8 °C.

CONCLUSION

In conclusion, net increases of *S. enteritidis* rarely occurred in either vitelline membrane or albumen isolated from eggs stored over an 8 week period. However, *S. enteritidis* populations were usually detectable and when net growth did occur in the different egg components, it usually occurred in the fractions isolated from older eggs. This response resembles the trends observed by Howard *et al.*^[24] for *S. typhimurium* inoculated onto egg components under similar experimental conditions. Previous studies with *S. enteritidis* have demonstrated that when it is introduced directly to yolk material, rapid growth is experienced^[27,34-36]. Therefore, if *S. enteritidis* was able to penetrate the vitelline membrane in the current study, counts from yolk samples should have been much higher. Consequently, even though vitelline membrane deterioration may have occurred, it was apparently not severe enough in this study to produce large increases in *S. enteritidis* yolk counts or high frequencies of eggs exhibiting *S. enteritidis* net growth. This is supported by the reports that rapid refrigeration of eggs and long term storage at refrigeration temperatures limits growth and penetration of *S. enteritidis* into egg contents^[32,37-39]. However, since *S. enteritidis* was generally detectable after inoculation of the albumen and vitelline

membrane at each weekly interval there still exists opportunity for multiplication to occur if internal egg environmental conditions suddenly become growth permissive.

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