ANNEX B

Distribution of *Salmonella* Prevalence in Hens and Eggs

CONTENTS

INTRODUCTION

This annex addresses the first stage of a farm-to-fork quantitative risk assessment designed to model the human-health risk attributable to *Salmonella*-contaminated eggs. It provides data analysis and support for modeling the percentages of *Salmonella*-positive eggs produced by *S.* Enteritidis (SE)-infected flocks (defined as hens that could become infected due to the presence of SE in the environment, as measured by the National Animal Health Monitoring System (NAHMS)[1] by a vertical or through shell route of contamination. These percentages may depend upon several biological and husbandry factors; therefore, a probability designed national survey of flocks would be needed to estimate the distribution of the percentages of contaminated eggs. However, as no such survey has been conducted, it is necessary to model the distribution in an indirect fashion by considering various data sources.

To model the percentages of *Salmonella*-contaminated eggs in the U.S., the percentage of flocks with *Salmonella* was modeled; the percentage of *Salmonella*-infected hens in an infected flock was then modeled; and the percentage of eggs contaminated with SE by transovarian contamination, or *Salmonella* on the shell was modeled. The product of these three percentages provides an initial estimate of the likelihood that an egg is *Salmonella*-contaminated. To account for the change in likelihood of contamination due to time of molting relative to egg laying, the weekly contamination rate per egg was multiplied by a molting factor each week post-molt for 10 weeks. The location of the contamination within the egg was considered to allow for subsequent differential SE growth rates based on the location of the initial contamination. For the case of eggshell contamination by *Salmonella* spp., the percentage of eggs that become *Salmonella* spp. contaminated by through shell penetration was modeled.

This annex provides data analysis and support for modeling:

1) The prevalence of SE and *Salmonella* spp.-infected flocks in the U.S.
2) The distribution of the percentage of SE and *Salmonella* spp.-infected individual hens within a flock.
3) The prevalence, near the time of lay, of SE-positive eggs produced by SE-infected hens in SE-infected flocks by a transovarian contamination.
4) The prevalence of *Salmonella* spp.-positive eggs produced by *Salmonella* spp.-infected hens in *Salmonella* spp.-infected flocks by through shell penetration contamination.
5) A weekly molting factor to capture the likelihood of contaminated eggs being laid by SE-infected molted hens for 10 weeks.
6) The percentages of contamination sites within an egg:
    a. In the yolk (*Ey*)
    b. On the vitelline membrane (*Ev*)
    c. Near the yolk but in the albumen (*Eac*)
    d. Farther away from the yolk but in the albumen (*Eaf*)
    e. In the inner shell membranes (*Es*)
    f. On the outer egg shell

The primary outputs of this annex are probability distributions to estimate the likelihood that an egg produced by either of two routes of *Salmonella* transmission under molted or non-molted status is SE-contaminated. That is, one distribution for each of the three conditions below:

1) SE-infected molted flocks via transovarian contamination.
2) SE-infected non-molted flocks via transovarian contamination.
3) SE-infected non-molted flocks via shell penetration.

THE DATA

Data in this annex were acquired by web-based electronic searches. References from relevant articles were assessed to acquire additional journal and book publications. Raw and unpublished data were obtained by direct correspondence with investigators and expert opinion was used. Data were analyzed by a weight of evidence approach: scientific publications were analyzed and interpretations made based on a preponderance of the evidence.

The estimate of the percentage of SE-infected flocks nationally was based on SE environmental sampling data from NAHMS.[1] In addition, the USDA National Agricultural Statistics Services (NASS)[2] and Pennsylvania SE Pilot Project[3] data were used as weights to account for regional SE prevalence differences and environmental false-negative sampling, respectively.

From the population of infected flocks, the distribution of the within-flock percentage of infected hens that would be laying SE-contaminated eggs was determined. Though there were no direct data for estimating this distribution, results from a 1991 and 1995 spent hen survey[4] were used as proxy for the percentage of hens in the laying hen population infected and potentially producing SE-contaminated eggs.

To determine the prevalence of SE-positive eggs produced by SE-infected hens in SE-infected flocks by a transovarian contamination, the within-flock prevalence was multiplied by the percentage of SE-contaminated eggs, the latter of which was estimated from eggs collected from experimentally inoculated hens over an 8-week period.[5] Additionally, as molting is known to increase the percent of SE-contaminated eggs laid by infected hens, data from SE-positive eggs collected after molting were used to determine weekly molting factors for 10 weeks post-molt.[3]

The percentage of *Salmonella*-contaminated eggs produced by through-shell penetration was modeled in a similar fashion for that of transovarian contamination. Spent hen surveys were used to determine the percentage of *Salmonella* spp.-infected flocks and to estimate the within-flock prevalence of *Salmonella* spp. An experimentally infected hen study was used to determine the percentage of surface contaminated eggs and the percentage of through shell contaminations was determined using data from Schoeni et al.[6] These analyses provided the number of transovarian or shell penetrated contaminated eggs produced by a molted or a non-molted infected flock, which were then inputted to the exposure assessment and risk characterization.

From the population of contaminated eggs, we sought to determine the distribution of contamination sites from through shell penetration or transovarian transmission. Identification of these percentages relative to one another is important as the site of contamination influences the subsequent growth rate in the egg. The growth of SE within the egg is a principle risk factor for consumers.

An extensive review of the published literature was prepared to investigate the factors that would influence numbers and levels of egg contaminations with SE. A discussion of the usefulness of data obtained from experimentally inoculated hens compared with those from naturally infected hens is given in attachment B1. The data and information presented in this annex were used to formulate assumptions and construct models throughout the risk assessments. A summary of these models and assumptions is given below.

Proportion of SE-Positive Flocks

Estimation of the number of SE-positive eggs in the U.S. begins with an estimate of the proportion of flocks in the U.S. that are SE-positive. An SE-infected flock is defined has having one SE-positive sample and assumed to have at least one SE-positive hen. The presence of SE within a flock varies in the U.S. due in part to husbandry issues such as rodent index, production house temperature and humidity, ventilation, stocking density, caging and feeding/watering systems. Though these issues will not be discussed further in the risk assessments, they serve to demonstrate that variability of SE among flocks is expected. What follows is a discussion of the data used to estimate the national percentage of SE-positive eggs.

NAHMS Layers '99 Survey

In 1999, NAHMS conducted a survey to estimate the prevalence of SE in layer flocks from 15 selected U.S. states.[1] Environmental sampling was conducted from May 3rd through October 22 in 200 layer houses. These 200 houses resided in 15 states and represented over 82% of the 1997 laying hens in the U.S. One house per farm was typically chosen at random for environmental sampling. At larger farms, multiple houses were sampled. Five manure samples, five egg belt samples, five elevator samples, and two walkway samples were gathered for each house (two swabs per sample). These samples were then shipped on ice for culturing to the Agricultural Research Service in Athens, GA. The survey estimated approximately 7.1% of the flocks in the 15 selected U.S. states were positive for SE with a standard error of 3.6%. This large standard error reflects the limitations of small sample size. A regional analysis of the sample results is presented in Table B1.

TABLE B1 NAHMS RESULTS FROM 1999 NATIONAL SURVEY USING ENVIRONMENTAL DRAG SWABS.

Region	% Flocks SE-positive	% U.S. Flocks In Region
Great Lakes	17.2 (13.7)	35%
Southeast	0.0 (--)	15%
Central	9.0 (7.2)	28%
West	4.4 (2.5)	22%
Total	9.6 (5.2)	N/A

Adjusting for regional differences

A 1999 USDA-National Agricultural Statistics Services (NASS) survey[2] was used to identify the percentage of total U.S. flocks by region, regardless of SE status. Using these percentages as weights, the NAHMS national estimate of SE-positive flocks[1] was adjusted to 9.6% with a

standard error of 5.2%. The addition of the NASS data therefore allowed for a more accurate national estimate of SE-positive flock prevalence.

Adjusting for false-negative test results

Environmental sampling can underestimate the percentage of SE-positive flocks due to false-negative results and low levels of SE shed by infected birds. An adjustment for false-negative results was made using data from a field trial conducted by Schlosser et al.[3] For environmental swab sampling, about 48% of infected flocks were positive on a single test. A single flock test usually consisted of collecting separate swab samples from each manure bank (typically 6 samples per flock), each egg belt (typically 6 per flock), and other surfaces in the poultry house (typically four samples from walkways or walls). In the field trial, 12 flocks' environments were sampled weekly for 12 consecutive weeks. Eight of the flocks had at least one positive test result during the 12 weeks of sampling. Among these eight flocks, there were 46 positive results from 95 environmental collections; apparently one test result was missing. Assuming these eight flocks were SE-positive for all 12 weeks, the above result implies an approximate 50% false-negative rate. Consequently, the proportion of positive flocks in the NAHMS study was multiplied by a factor of approximately 2 (95/46) to adjust for underestimation of SE-infected flocks based on false-negative test results.[a]

Flock prevalence estimate

The proportion of SE-infected flocks in the U.S. was estimated at 7.1% with a standard error of 3.6% based on the NAHMS survey results. This proportion was adjusted for regional differences, 9.6±5.2%, and then multiplied by a factor of two to account for false-negative test results. Consequently, the prevalence of SE-infected flocks is assumed 19.2% with a standard error of 10.4%.

SE-infected Birds in an SE-positive Flock

Given the proportion of SE-infected flocks as estimated above, the next task was to estimate the proportion of birds in an SE-infected flock that were SE-positive. The number of individually infected hens within an infected flock is likely to differ among flocks by region and season. This variation could be due to differing rates of SE transmission among birds within an infected flock. Factors affecting this are likely to be conditional on hen and SE strain genotype variability. Environmental and husbandry factors such as rodent index, production house temperature and humidity, ventilation, stocking density, caging and feeding/watering systems will also alter transmission rates. Additionally, mitigation strategies such as vaccination and competitive exclusion have been used to lower the likelihood of intestinal colonization by *Salmonella* spp.,

[a] To apply the false-negative rate of Schlosser et al.[3] to the data from the NAHMS survey,[1] testing procedures were evaluated for both studies. The sampling and culturing procedures employed by Schlosser et al. are somewhat comparable to that used in the NAHMS survey. Therefore, the false-negative rate of Schlosser et al. was applied to the regionally adjusted NAHMS survey estimation of SE-infected flocks.

and therefore reduce shedding of these bacteria. Because many factors affect the proportion of infected hens within a flock, variability is expected among flocks.

Prevalence of SE in spent hens

To estimate the proportion of birds in an infected flock that were SE-positive, two national studies utilizing spent hens[2] at the time of slaughter in 1991[77] and 1995[8] were used. These studies are the only national surveys we know of that attempt to quantify SE within-flock prevalence.

The use spent hens to estimate the SE within-flock prevalence of younger laying hens is uncertain. Ebel et al.[7] said, "Because the bird samples in this [the 1991 spent hen] survey were at the end of production, it is uncertain whether these results represent recent colonizations acquired during transport to slaughter or chronic colonizations acquired earlier in production, or whether over time a house of birds will accumulate a certain prevalence of colonization." Therefore, even though the spent hen survey data is applicable to older hens at the time of slaughter, the usefulness of spent hen survey data to predict the likelihood of commercial within-flock SE prevalence is unclear.

For instance, variation in within-flock prevalence is expected due to the dynamic nature of SE; however, the spent hen surveys indicated most SE-positive flocks have relatively few SE-positive hens. Seventy-seven flocks had one positive sample test among the average of 58 tests per flock and 247 SE-positive flocks. This suggests 31% (77/247) of the infected flocks had low within-flock prevalence. The highest number of positive tests was 44 for 1 flock out of 247 SE-positive flocks, suggesting 0.4% (44/247) of SE-positive flocks have high within-flock prevalence.[7;8] However, the number of tests per flock is uncertain. If this number were low due to missing test samples, this would imply greater within-flock prevalence. Therefore, depending upon the number of samples tested for each flock, the estimate of the percentage of infected hens could be higher. Factors that could influence the estimated within-flock prevalence of hens are discussed below.

Age of spent hens

Spent hens are more likely to be older than hens used to produce eggs. Therefore, the hens used in the 1991 and 1995 spent hen surveys will be birds about 2 years of age. The age of spent hens suggest they will be physiologically different from hens of laying age. This physiological difference might affect the within-flocks prevalence of SE.

Hens can consistently produce eggs at a normal rate for about 45 weeks. This is followed by a decline in egg production that varies with hen breed. Producers molt their hens once at 45 weeks of age, a procedure that rejuvenates the egg-laying rate. Post-molt, hens are often kept for egg production until they are 100 weeks old; some producers molt their hens a second time at 100 weeks. This depends on the current market. Hens are occasionally kept for 120 weeks. Consequently, spent hens might be between 1 and 2.5 years olds. Most spent hens will be about 2 years of age because the majority of production houses molt their hens once.

Prevalence of *Salmonella* spp. in Laying Hens

The text suggests the prevalence of *Salmonella* spp. in spent hens is very high, implying non-SE *Salmonella* might be more competitive in spent hens. For this to be plausible, the prevalence of *Salmonella* spp. in hens of laying age must be lower. However, no U.S. studies were identified investigating the prevalence of *Salmonella* spp. in naturally infected flocks of laying age to determine the baseline frequency of *Salmonella* spp. in these hens.

An article identifying *Salmonella* spp. from the wash of chicken carcasses and raw ground chicken was used to determine a baseline for the presence of *Salmonella* spp. in broiler chickens.[9] These authors reported that chickens can harbor many different *Salmonella* serotypes, and as an upper bound, they found 26.2 and 30.0 % of chicken wash and raw ground chicken contaminated with *S.* Heidelberg (among other *Salmonella* serotypes), respectively. However, these percentages are difficult to compare directly with the spent hen surveys as these birds are broilers and have gone through levels of processing that might contaminate samples.

Two Canadian studies identified layer flocks as most often contaminated with *S.* Heidelberg at frequencies of 20 and 10%.[10;11] These two studies are difficult to compare directly to the spent hen surveys as differences in Canadian production might affect the epidemiology of *Salmonella*. In addition, these studies assayed hen fecal droppings and other environmental samples that might underestimate the prevalence of *Salmonella* compared with cecal samples.

These data suggest the baseline of commercial hens infected with *Salmonella* relatively low compared with those in spent hen surveys. This difference in prevalence is likely due to the increased susceptibility of older[12] and molted hens[13] to infection.

Susceptibility to SE and competing Salmonella *spp.*

To explain why so many infected flocks have so few SE-infected hens as determined by the 1991[7] and 1995[8] spent hen surveys, the effect of hen age on egg colonization by SE was analyzed. It appears older hens have weakened immune systems, making them more susceptible to colonization by SE.[12] Studies suggest that the antibody level produced by an immune response of a 62-week old hen declines more quickly than that of a 37- or 27-week-old hen. It is likely that older hens will be more susceptible to SE infection for longer periods due to the inability to mount or sustain a 'normal' immune response. Molted hens are more susceptible to SE intestinal colonization and prolonged fecal shedding as compared to non-molted hens.[14;15] These data imply spent hens are more susceptible to SE infection and spent hens might overestimate SE within-flock prevalence. On the other hand, spent hens might underestimate prevalence due to the presence of competing *Salmonella* spp.

To investigate the implications of competing *Salmonella* spp., surveys were sought that elucidated the baseline prevalence of *Salmonella* spp. in commercial laying hens. However, no such survey was found (see textbox). We thus used the spent hen data. Spent hen surveys observed a large percentage of flocks are frequently colonized with other *Salmonella* serotypes besides SE: 76.2, 97.4, 86, 98 and 100% respectively as determined by pooled samples of ceca[b] or ovaries.[7;8;16-18] Only 1 of the studies serotyped non-SE *Salmonella* and found that as an upper bound, 56.5% of the hens were colonized with *S.* Heidelberg.[17] In addition, these surveys identified only 2.4, 1.5, 3.0, 5.1, and 0.16% flocks as SE-positive. It appears that spent hens are infrequently colonized with SE, yet may be frequently colonized with non-SE *Salmonella*.

[b] Closed intestinal pouches connected to the hen lower intestinal tract.

Based on these findings it seems spent hens are likely more susceptible to many *Salmonella* serovars, not only SE. In the presence of competing *Salmonella* strains, this might have the effect of other *Salmonella* serotypes out-competing SE for the same niches within a hen. Therefore, SE might be under represented in spent hens. This could explain why the two spent hen surveys had low SE within-flock prevalence for spent hens.

False-negative rate of spent hen survey

The 1991 and 1995 spent hen surveys might have underestimated the within-flock prevalence due to false-negative recovery rate of SE. This is evidenced by the results of Waltman et al.[18] Of the 6 SE-positive samples identified in this study, 3 isolates were recovered on XLT-4 plates, 5 on BGAN plates, and 1 by the extended incubation method (see textbox). That is, from the 6 known SE-positive samples, use of XLT-4 failed to recover isolates from 50% of the samples. SE levels in the samples were not known, but the levels in some of the missed samples were sufficiently high to be detected by the BGAN. Ebel et al.[7] and Hogue et al.[8] utilized XLT-4 plating to identify SE within pooled cecal samples and could have failed to detect some SE-positive samples. Miller et al.[19] state, "It is suggested that two different types of plating media be inoculated to further reduce the possibility of a false-negative finding that could occur if a particular strain of *Salmonella* were sensitive to an inhibitor used in one of the two media."

Additional evidence to support a false-negative sampling rate of the 1991 and 1995 spent hen surveys is given below. Analysis of the ceca, as performed in the 1991 and 1995 spent hen surveys is a good indicator of hen infection by SE,[20-22] as positive cecal culture samples are typically the most frequent when other extra-intestinal tissues are cultured simultaneously. Though it is unclear how hens typically become SE-infected, it is generally thought hens are horizontally infected through ingestions of contaminated feed and water, or through contact exposure and subsequent preening. Even airborne infection has been shown to result in some direct oral contamination.

Protais et al.[22] showed that at 28 days post-inoculation, 1 experimentally inoculated hen out of 16 was infected in the liver, spleen, and oviduct. None of the hens was SE ceca-positive. Using a different hen line, these authors demonstrated 9 hens out of 10 were ceca-positive; yet SE was present in the spleen and the ovary in the 1 ceca-negative hen. In addition, Keller et al.[20] found in 1 experiment that SE was detected in 70.0% of experimentally inoculated hens by culture of a ceca and small intestine pool; yet organ (heart, spleen, liver and gallbladder) culture of these same hens identified 95.0% as infected with SE. Assuming the false-negative recovery rate is very low, these data suggest that although culture of ceca is a reliable indication of hen infection, a small percentage of hens will be ceca-negative but colonized with SE. [20-22]

Waltman et al.

To estimate hen flock prevalence of SE, Waltman et al.[18] pooled ceca from spent hens and incubated the samples in rich medium (TT) for 24 hrs. The culture was then inoculated onto either xylose-lusine-tergitol-4 (XLT-4) plates or brilliant green agar supplemented with 20 µg novobiocin/ml (BGAN). XLT-4 and BGAN plates identified 64% (1536/2418) and 72% (1740/2418), respectively, of the total *Salmonella*-positive cecal samples (82% (1993/2418) together). If these procedures were negative, an extended incubation in TT broth was then performed and streaked onto the two plate types. This latter method identified 425 more samples that were positive. XLT-4 medium was designed for recovery of Group D *Salmonella* (including SE) where BGAN can be used to identify a broader range of *Salmonella* serotypes.

Data Analysis of Spent Hen Survey

Estimating within-flock prevalence

Table B2 presents a compilation of the data from the two spent hen surveys.[7;8] Three hundred hens were sampled from each flock and for each hen, 1 cecum was examined. Five ceca were pooled and analyses were performed on the pooled samples. Ebel et al.[7] reported that on average 58 samples per lot were analyzed from 406 lots. The data in Table B2 show that the number of flocks for which only 1 or two pooled samples were positive is relatively large. The mode of the distribution of the number of positive samples is 1, suggesting that for most flocks a relatively small percentage of hens would be infected. The largest number of positive samples is 44. Let q be the fraction of positive samples within a lot, and h be the false-negative rate, then an estimate of the percentage of hens infected, $\hat{p}(q)$, in a flock is given by:

$$\hat{p}(q) = 1 - \left[1 - \frac{q}{1-h}\right]^{1/5} \qquad \text{(B1)}$$

If $h = 0.15$ and $q = 44/58$, corresponding to 58 samples for the flock, then $\hat{p}(q) = 36\%$.

TABLE B2 DATA FROM THE SPENT HEN SURVEYS[7;8] TAKEN FROM THE 1998 FSIS SE RISK ASSESSMENT.[4]

Number of Positive Pooled Samples	Number of Flocks	Estimated Within Flock Percentage of Infected Hens
0	464	0.00
1	77	0.41
2	39	0.82
3	23	1.25
4	18	1.68
5	9	2.12
6	6	2.56
7	8	3.02
8	7	3.48
9	8	3.95
10	4	4.43
11	6	4.92
12	4	5.43
13	4	5.94
14	2	6.46
15	2	7.00
16	6	7.55
17	1	8.11
18	3	8.69
19	3	9.28
21	2	10.51
22	3	11.15
23	1	11.81
24	1	12.49
25	1	13.19
26	2	13.92
27	2	14.67
28	1	15.45
36	1	23.05
39	1	26.89
42	1	31.75
44	1	35.98

[a]Entries are number of positive pooled samples (of 5 ceca), number of lots with this number of positive samples, and an estimate of the within-flock percentage of infected hens, computed, assuming a false-negative rate of 15% and 58 samples analyzed per flock.

Let p be the percentage of infected hens within a flock, and assume that the distribution of p is f. The probability of a positive result on a sample, given p and h, is $q(p) = (1- (1-p)^5)(1-h)$, so that the probability distribution of x positive samples, $b(x|p, n)$, from n tests would be a binomial distribution with parameters n and $q(p)$. Let k_x be the number of flocks with x positive tests, and consider the following measure: $E_x = k_x/(1-b(0|\hat{p}(x, n))$ - the number of flocks with x positive samples divided by an estimate of the probability of at least 1 positive finding from a flock for which x positive findings were observed. In some rough sense, E_x is an estimate of the number of flocks in the population for which the expected number of positive samples would be x. Thus, for example, E_1 is an estimate of the number of flocks for which it would have been expected to detect 1 positive from n samples.

To visualize the shape of the distribution of the percentage of hens that are infected within infected spent hen flocks, an estimate of the cumulative distribution function, $F(p)$, for $p> 0$, can

be obtained by considering the E_x values. For each x, there is a corresponding percentage of hens infected in the flock, $p(x)$. The cumulative distribution function, $F(p)$, is estimated as:

$$\hat{F}(p) = \frac{\sum_{x:p(x)LEp} E_x}{\sum E_x} \qquad \text{(B2)}$$

Figure B1 is a plot of the log-log transformation: $t = \ln(-\ln(1-F(p(x)))$ versus $\ln(p(x))$. As is evident from the plot, the data points fall on a straight line, given by: $t = a + b\ln(p)$, where $a = 2.2736$ and $b = 0.5272$. This pattern suggests that a Weibull distribution be used to estimate F.

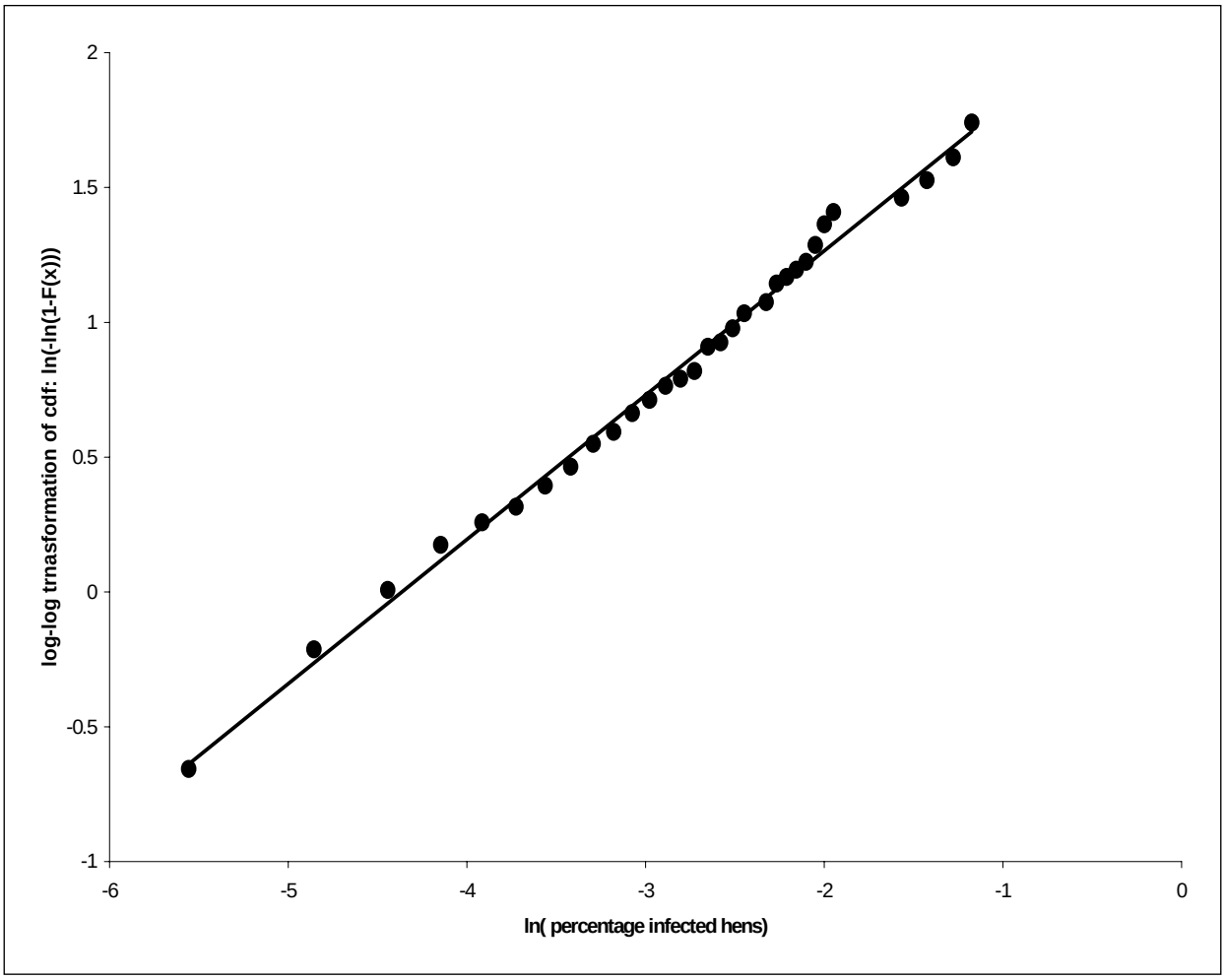

FIGURE B1 PLOT OF LN(-LN(1-$F(P)$)) VERSUS LN(P) WHERE $F(P)$ IS ESTIMATED FROM EQUATION B2.

Taking the inverse transform of t, it is derived that the cumulative distribution function F is approximated as a Weibull distribution: $W(p)$, given by

$$W(p \mid b,c) = 1 - e^{-(p/c)^b} \tag{B3}$$

where b = 0.5272 and c = exp(-a/b) = 0.01340.

The above estimates do not account explicitly for the flocks with low percentages (< 0.33%) of infected hens - flocks likely to be counted as SE-negative. A more formal estimate, using a maximum likelihood estimation procedure, is made by assuming that the distribution F with density function f is such that for p>0, $f(p)$ depends upon parameters of θ, and $f(0)$ is a parameter to be estimated. Thus, based on the above analysis, assume that $f(p)$ is a Weibull distribution with parameters $\theta = (b, c)$. For a given test (a sample of 5 ceca), let $q(p) = [1 - (1-p)^5](1-h)$ be the probability of a positive result. Then, the probability of x positive out of n tests (for a flock) is a binomial distribution with parameters $q(p)$ and n. The likelihood of observing x positive tests, from a total of n tests is

$$L\left(x \middle| b, c, f(0)\right) = f(0)\,(1 - \delta_{>0}) + \delta_{>0}\,(1 - f(0)) \int_0^1 \binom{n}{x} q(p)^x\,(1 - q(p))^{n-x}\, f(p \middle| b, c)\,dp \tag{B4}$$

MLE estimates of the parameters of Equation B4 were determined using Newton-Raphson iteration. The actual estimates were made on transformed values: $\mu = \ln(c)$ and $s = -\ln(b)$, to avoid boundary problems. Convergence was obtained, with a value of $f(0)$ equal to 28.5%. The MLE estimates of the other parameter values of the Weibull distribution were b = 0.43015 and c = 0.005389. Table B3 gives observed and predicted numbers of samples for given numbers of found positive samples.

TABLE B3 OBSERVED AND PREDICTED NUMBER OF SAMPLES BASED ON MLE ESTIMATES.

No. positive samples per flock	Observed no. flocks	Predicted no. flocks
0	464	464.0
1	77	71.3
2	39	38.6
3	23	25.2
4	18	18.1
5	9	13.7
6	6	10.8
7	8	8.7
8	7	7.2
9	8	6.0
10	4	5.1
11-19	31	25.0
20-52	17	16.9
Totals	711	710.7

The MLE estimates of *:* and *s*, together with standard errors and correlation, are given in Table B4.

TABLE B4 MLE ESTIMATES OF PARAMETERS, *:* AND *S*, WHERE *B* = EXP(-*S*) AND *C* = EXP(*:*) ARE PARAMETER VALUES FOR WEIBULL DISTRIBUTION: *W*(*P*) = 1-EXP(-(*P*/*C*)*B*), WHERE *P* IS THE FRACTION OF INFECTED HENS IN A SPENT HEN FLOCK.

	:	*s*
Estimates	-5.22345	0.84363
Standard Errors	0.36309	0.10775
Correlation		-0.91281

Using these values, the estimate of the 99th percentile is 0.188 with a 97.5% upper confidence bound equal to 0.255; the estimated 99.9th percentile is 0.482, with a 97.5% upper confidence bound of 0.706.

Estimating the false-negative rate

As discussed above,[18] from 6 known SE-positive samples, 3 were not detected positive by the methodology used in the spent hen survey. While a 50% false-negative rate may be high, such a rate cannot be dismissed, particularly for low level SE-infected flocks. It is possible that the false-negative rate would be a function of the percentage of positive test – a higher percentage would imply higher levels of SE, generally, which would imply a lower false-negative rate. No information on this is available, and thus, for simplicity, a moderate false-negative rate of 15% was assumed in the above analysis.

Proportion of SE-positive Eggs

The purpose of this section is to estimate the percentage of SE-positive egg produced by SE-infected molted and non-molted flocks via transovarian contamination, i.e. vertical transmission. These estimates of the numbers of infected shell eggs are used in the Exposure Assessment and Risk Characterization. As discussed above, some flocks and birds are SE-infected. Infected birds can lay SE-positive eggs. These eggs are infected via transovarian contamination. Birds can also lay SE free eggs. These eggs may remain SE free or they can become infected via through shell penetration. To estimate the percentage of eggs laid by transovarian contamination, data on the number of eggs produced by hens experimentally infected over an 8-week period were evaluated. This percentage (q) is multiplied by the percentage of SE-positive hens (p) to estimate the percentage of SE-positive eggs produced by SE-positive non-molted hens. As molting will increase this percentage, weekly molting factors were developed and applied to the percentage of SE-positive eggs per week post inoculation to estimate the percentage of SE-positive eggs produced by SE-positive molted hens. p_1 (% of SE+ hens 1 week post-molt) q_1 (% of SE+ eggs 1 week post-molt) m_1 (molting factor 1 week post-molt) = $p_1q_1m_1$ (% SE+ eggs produced by 1 week molted hens by transovarian contamination). Data and analysis of how these percentages were estimated is given below.

EGG CONTAMINATION OVERVIEW

SE contaminate the internal contents of eggs by two modes of transmission: transovarian contamination and through shell penetration.

Transovarian Contamination of Eggs

Transovarian contamination appears as the primary route of SE egg contamination. Several studies have isolates SE from ovaries and oviducts of naturally and experimentally infected hens.[20;21;23-27] The presence of SE in the reproductive tract was consistent with the production of SE contaminated eggs in the albumen, the yolk or both. Several studies examining naturally and experimentally infected hens failed to show a strong correlation between SE egg shell contamination and contamination of internal egg contents,[21;28;29] suggesting transovarian contamination. This risk assessment focused on the percentage of SE-positive eggs produced by transovarian contamination to calculate the percentage of SE-positive eggs produced by an SE-positive flock.

The proportion of SE-positive eggs produced by transovarian contamination is estimated using data from a study of SE-positive egg production by experimentally inoculated hens over an 8-week period. Then the effect of molting on SE-positive egg production is considered through the development of a factor that can be applied to the proportion of SE-positive eggs. The analysis concludes with a discussion of the possible sites where SE can be deposited within the egg because this is important to future growth of the bacteria.

Mechanisms of transovarian contamination

Transovarian contamination occurs when SE reside in the reproductive tissue of an infected hen and are transferred to the internal compartments of the egg during the egg's formation. Infection of the hen's reproductive system is necessary for transovarian contamination. Estimating the percentage of transovarian-contaminated eggs laid by an SE-positive hen is important for subsequent estimates of the frequency of the different types of SE contamination in a shell egg, which in turn is important because different types of contaminations result in different rates of growth of *Salmonella* in the egg and different numbers of bacteria per egg. The number of bacteria in an egg is important in estimating the effectiveness of pasteurization as well as the risk of illness to humans.

Different experimentally inoculated hen breeds and SE strains have been used to qualify ovary and oviduct SE infection (Table B5). The estimate of the percentage of SE-positive eggs contaminated via transovarian contamination begins with evidence of SE colonization of the ovary and oviduct and the level of SE found within these tissues.

<u>SE colonization of the ovary and oviduct</u>

A high percentage of the ovaries and the oviducts of hens inoculated with SE are colonized by SE within days of inoculation.[20;24;26;27] Colonization sustainability, i.e., SE persistence over time, of reproductive tissue was not maintained at the initial prevalence (Table B5),[20;21;27] though it is

possible SE levels below the culturing detection limit produced false-negatives. Nevertheless, the infection appears dose-dependent (Table B5).[23;30]

Gast[23] found no SE-positive reproductive organs with a 4 $\log_{10}$ cfu/hen inoculum, yet 3.8% (3/80) pooled egg contents samples were positive for SE, respectively, supporting the possibility of false-negative results. Additionally, Keller et al.[20] found 0% (0/34) reproductive tissue SE-positives at 3 weeks, yet 3.6% (3/84) SE-positive pooled egg contents samples. Therefore, though the magnitude of reproductive tissue infection decreased over time to non-detectable levels, hens still would be capable of producing SE-contaminated eggs. These data suggest SE ovary or oviduct colonization can be below the level of culturing detection, yet could still contain sufficient numbers of SE to contaminate an egg.

TABLE B5 PERCENTAGE OF DOSED BIRDS WITH SE COLONIZATION OF THE OVARY AND OVIDUCT[A]

Publication	Dose	Days post-oral inoculation						
		2-4	4	7	14	9-21	32-42	154
Analysis of combined ovary and oviduct								
Thiagarajan et al.[26]	8 $\log_{10}$ cfu/hen		28.6% (10/35) hen+					
Keller et al.[24]	8 $\log_{10}$ cfu/hen		39.4% (26/66) hens+					
Separate analysis of ovary and oviduct								
Keller et al.[20]	8 $\log_{10}$ cfu/hen	100% (6/6) ovary+; 67% (4/6) oviduct+		33% (2/6) ovary+; 13% (1/8) oviduct+		0% (0/33) ovary, oviduct+	4.2% (1/24) ovary, oviduct+	
Gast and Beard[31]	9 $\log_{10}$ cfu/hen			70% (14/20) ovary+; 60% (12/20) oviduct+	4% (1/24) ovary+; 13 (3/24) oviduct+			8% (3/40) ovary+; 5% (2/40) oviduct+
Gast[23]	4 $\log_{10}$ cfu/hen				0% (0/40) ovary, oviduct+			
	6 $\log_{10}$ cfu/hen				10% (4/39) ovary+; 5% (2/39) oviduct+			
Timoney et al.[27]	6 $\log_{10}$ cfu/hen		67% (2/3) ovary+; 100% (3/3) oviduct+	100% (3/3) ovary+; 67% (2/3) oviduct+		0% (0/5) ovary, oviduct+	0% (0/10) ovary, oviduct+	

[a]Hens were dosed with SE, sacrificed, and the reproductive organs removed for analysis of SE. Blank cell indicates no sampling.

SE colonization of the ovary

Okamura et al.[32] found the ovarian preovulatory follicular membrane (capillary-rich sac surrounding the yolk within the ovary) SE-positive 87% (21/24) of the time. However, Thiagarajan et al.,[25] who separated the follicular membrane from the yolk, found 57% (8/14) SE-positive follicle membranes; but only 21% (3/14) yolks (with vitelline membrane) from these follicles were SE-positive. This suggests SE colonization of the ovary need not result in yolk contamination (see textbox). The risk assessment includes the assumption that though the ovary can be colonized frequently, yolk contamination (*Ey*) is less frequent.

SE colonization of the ovary and egg yolk contamination (*Ey*)

Experiments isolating SE from the hen ovary demonstrate the ovary can be frequently contaminated.[20;21;27;32] However, Thiagarajan et al.[25] demonstrated that when yolks were removed by cutting open the follicle and letting the yolk fall into a container, the follicle membrane was more frequently SE-positive than the yolk, suggesting even though components of the ovary are SE-infected in a high percentage of hens, the yolk and the vitelline membrane appear to be infected at a lower frequency.

Thiagarajan et al.[25] suggest an explanation for this apparent contradiction. SE can contaminate the granulosa cells of the follicle membrane. During ovulation, the follicle stigma ruptures, releasing the yolk, surrounded by the vitelline membrane, into the oviduct. Then, SE colonized/invaded-granulosa cells could "slough off," onto the yolk,[25] perhaps resulting in contamination of the vitelline membrane (*Ev*) or internal yolk contents (*Ey*). This would explain the high frequency of observed ovary infections, but low frequency of fresh inner yolk contents with SE.[33]

SE colonization of the oviduct

When the oviduct was subdivided into infundibulum, magnum, isthmus and uterus,[c] SE-positive cultures were observed at similar frequencies throughout the oviduct (Table B6).[20;32;34] However, Keller et al.[20] found the frequency of SE-positive cultures from the upper magnum was greater than any other oviduct tissue (15% vs. 2.5-5%) in 1 of 3 experiments using a different hen breed.

General colonization of the oviduct implies a greater likelihood of albumen contamination far from the yolk (*Eaf*) compared to close to the yolk (*Eac*), as the majority of albumen is composed of outer albumen and exposed to the oviduct for longer periods. However, as Keller et al.[20] found, specific areas of the oviduct could be preferentially colonized depending on such factors as hen breed and SE strain. Preferential colonization of the upper magnum would probably lead to more *Eac* colonization. This is important as the location within the egg where SE is deposited could determine the frequency and magnitude of subsequent SE growth.

TABLE B6 LEVELS OF SE COLONIZATION WITHIN THE HEN REPRODUCTIVE TRACT.[32;34]

Tissue	3[a]	6	8.25[d]	9	12
Ovary	0.8[b] (2/5)[c]	0.4 (1/5)	E[e]-4.3 (9/9)	1.4 (2/5)	4.2 (5/5)
Infundibulum	1.0 (2/5)	0.6 (1/5)	0-3.7 (2/9)	1.2 (2/5)	4.0 (5/5)
Magnum	1.5 (2/5)	0.6 (1/5)	0-5.2 (2/9)	0.6 (2/5)	3.7 (5/5)
Isthmus	0.6 (1/5)	0.4 (1/5)	0-4.5 (5/9)	2.1 (2/5)	4.5 (5/5)
Uterus	0.8 (2/5)	0.6 (1/5)	0-4.7 (1/9)	0.4 (1/5)	4.0 (5/5)

[a] Age of hen (months) when SE inoculated.
[b] Log_{10} SE/g.
[c] Positive samples of total assayed.
[d] Okamura et al.[32;35]
[e] SE detected below enumerable level.

[c] The oviduct is divided into four sections. The infundibulum is the oviduct opening. The magnum and the isthmus provide albumen and the inner shell membranes for the egg, respectively. The uterus lays down the outer shell.

Levels of SE colonization of the ovary and oviduct

Okamura et al.[32] and Hassan and Curtiss[34] measured SE levels within 4 functionally divided oviduct sections. These data indicate SE contamination of the oviduct can be considerable and extend the length of this organ. These data also suggest the hen's age affects the level of SE within oviduct tissue: older hens are more heavily colonized by SE (Table B6).

SE Within the Oviduct Likely Predicts Where SE is Initially Deposited Within the Egg

Contamination of the infundibulum, the opening to the oviduct, could yield *Ev* contamination. This site is where fertilization of the ovum (yolk) takes place, suggesting intimate contact with the yolk vitelline membrane. The yolk resides in this location for a half hour after which it moves to the magnum, where it travels from upper to lower magnum (3 hrs). Within this organ, dense albumen is first deposited about the yolk, then thin albumen, followed by dense albumen and thin albumen. Infection of the upper magnum could lead to *Eac* contamination and *Ev* contamination as the yolk enters this organ. However, as the majority of the albumen's volume would constitute an area that could harbor *Eaf* contamination, infection within the magnum would likely lead to more *Eaf* contamination compared to *Eac* or *Ev*. The yolk then moves to the isthmus, where the two soft-shell inner membranes are laid over the albumen (1 hr). At this point, SE could contaminate the inner shell membranes leading to *Es* contamination (see next section). *Eaf* contamination could occur at any point prior to complete deposition of inner shell membranes. The yolk then moves to the uterus where the outer shell and cuticle are deposited (20 hrs). The uterus moves the egg into the vagina followed by the cloaca. This latter organ is where the reproductive system joins the digestive system. The vagina and cloaca can be colonized by SE due to their proximity to the colon, potentially leading to SE shell contamination (*Ep*). *Ep* contamination, as discussed below, could occur after complete shell deposition until the egg is laid. The egg then passes through the vent, the opening that serves for both excretion and egg laying. Therefore, depending where SE is located within the oviduct, this might dictate the incidence of *Ey*, *Ev*, *Eac*, *Eaf*, *Es* and *Ep* contamination.

Summary of SE Colonization of the Ovary and Oviduct

Data suggest both the ovary and oviduct can be heavily contaminated with SE.[32;34] Simply having ovary-positive status does not predict egg contamination (see textbox).[20;23;24] The prevalence of hen colonization by SE diminishes over time to below detectable levels in the ovary and oviduct. It appears SE reproductive tract colonization can be below the level of detection, yet could still contain sufficient numbers of SE to contaminate an egg internally. The data also suggest different sites of infection within the oviduct lead to various SE localization within the egg.

ESTIMATING THE PERCENTAGE OF SE-POSITIVE EGGS BY TRANSOVARIAN CONTAMINATION

To estimate the percentage of SE-positive eggs produced by SE-infected hens, studies were identified that investigated the number of SE-positive eggs being produced by SE-infected hens. Studies were identified that followed infected hens for four weeks and, as in one study, for eight weeks. Because kinetics of infection, i.e. persistence of the organism within the hen, and their relation to continued SE-positive egg production is unclear, this 8-week-study was useful to

reveal the pattern of SE-positive egg production over 8 weeks. The percentage of SE-positive eggs produced in this study is assumed the percentage of SE-positive eggs produced by a SE-positive non-molted hen at any moment. Data analysis and support for this assumption is discussed below.

Typically, the course of an SE infection in a group of experimentally inoculated hens begins with a large frequency of birds fecally-positive for SE. Depending on the inoculation dose, these birds can quickly mount an antibody response that peaks within 1-2 weeks. The majority of SE-positive eggs are produced during this time. Once the antibody response has been established, fecal shedding of SE and production of SE-positive eggs decrease. These observations suggest formation of an immune response is important for reduction of internally colonized SE and production of SE-positive eggs. Gast and Beard[21] and Gast,[23] utilizing inoculums of 9 $\log_{10}$ and 6 $\log_{10}$ cfu SE, respectively, showed that the majority of SE-positive eggs was produced within 2 weeks of inoculation hens aged 62, 37 and 27 weeks.

Bichler et al.[5]

Bichler and colleagues examined SE-positive eggs produced by SE-positive hens over eight weeks. SE contamination was identified within four egg compartments: outer shell, inner shell membranes, albumen, and yolk. The SE inoculum dose administered to hens would be expected to be sufficient to infect all hens. Eggs were examined upon lay and recovery methods to isolate SE from egg compartments were acceptable. In addition, the hen serum antibody response and the SE fecal carriage were monitored during the course of the infection. Naturally infected hen studies were not used to identify the percentage of SE-positive eggs because of such unknown factors as the prevalence of SE infection within the flock and the presence of other *Salmonella* spp.

To estimate the percentage of SE-positive eggs produced by transovarian contamination, the risk assessment used data from a study by Bichler et al.[5] (see textbox). Twenty-five-week-old white leghorn hens were inoculated with 10 $\log_{10}$ cfu SE (this dose may lead to high SE levels in reproductive tissue; thus care should be exercised in interpreting the results.) Following inoculation, each egg produced by treated hens was cultured for SE within the albumen, yolk and the inner shell membrane compartments. This latter compartment, the inner shell membranes (IS), is located just beneath the outer shell and can be infected by transovarian contamination. This compartment represents an internal contamination site within an egg and was used in tallying the total SE-positive eggs. The IS contamination event (*Es*) is discussed below. Based on contamination of the albumen, yolk and IS, 52% (32/61) of the eggs were internally contaminated with SE during week 1. This percentage fell to 4% (22/531) during the remaining 7 weeks (Table B7). The average of SE-positive eggs over the 8 weeks was 8.62% (Table B7).

TABLE B7. ESTIMATING THE PERCENTAGE OF SE-POSITIVE EGGS.[5]

	Week 1[a]	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Total
Albumen+	28	1	0	0	0	0	0	0	29
Yolk+	28	2	1	2	0	2	3	2	40
Albumen and Yolk+	25	0	0	0	0	0	0	0	25
Inner shell membrane only[b]	1	0	2	0	5	0	1	1	10
Total (%) SE-positive eggs	32 (52)	3 (4.9)	3 (3.7)	2 (2.4)	5 (6.0)	2 (2.3)	4 (4.9)	3 (6.0)	54 (8.6)

[a]Weeks post-inoculation.
[b]See SE inner shell membrane contamination section.

Kinetics of SE-positive Egg Production by Transovarian Contamination

To predict SE-positive egg production post-8 weeks, trends of contamination and SE-positive egg production was examined. Fifty-two percent of the SE-positive eggs were produced within the first 7 days, then dropped to a steady rate of about 4.1%. The drop was preceded by a peak antibody response that declined 17 days post-inoculation, suggesting the immune response influenced the frequency of SE-positive egg production. By 8 weeks, 43% of hens still had detectable antibody responses. This serum antibody decline was followed by an increase in positive cloacal[d] samples, suggesting that with the decline of the antibody response, SE could more vigorously colonize the hen's intestines. This could serve to infect naïve hens as well as re-infect other hens by dissemination into the environment. This increase in cloacal positive samples was not followed by an increase in SE-positive eggs by 8 weeks.

The data presented above suggest a pattern of increased SE-positive egg production immediately after SE exposure, followed by a period of lower SE-positive egg production. It is unknown if this trend would extend beyond 8 weeks, as the frequency of SE-positive eggs remained steady without further decrease from 2-8 weeks (Table B7). This risk assessment is unable to predict the percent of SE-positive eggs produced following 8 weeks. However, the data do suggest that a cycling of SE infection might occur within a flock (see textbox). That is, even though a decrease in the immune response did not result in an increased frequency of SE-positive eggs by 8 weeks, it did suggest that 57% of the hens at the end of this experiment would be able to disseminate SE into their environment due to their lowered serum antibody levels. Newly infected hens produced SE-positive eggs at a high rate (52%).[5]

Infection Cycling in Naturally-Infected Flocks

The concept of cycling of SE infection within a flock is supported in part by studies of naturally SE-infected flocks. Humphrey et al.[36] observed hens typically laid SE-positive (SE+) eggs in a temporal pattern, suggesting a clustering effect of SE+ egg production. Three SE+ eggs were laid between Feb. 15-17, and 5 SE+ eggs were laid March 26 to 28. All hens produced only 1 SE+ egg, except for 1 hen that produced 2 SE+ eggs corresponding to those dates. In addition, single SE+ eggs were detected sporadically from three hens between the start and end of the experiment (March 12, April 7, 16). The time between the two observed clusters was 41 days. Clustering could represent recent infection in hens or re-infections that resulted in SE+ egg production due to the lack of a quick adaptive immune response; times when hens are more stressed and therefore more susceptible to SE primary infection, or re-infection; or low-level colonized hens unable to maintain equilibrium with SE due to stress. Stress due to production could have a synchronizing effect on SE+ egg production.

These data may reflect a natural cycling of transmission/contamination, where more SE+ eggs will be produced by a flock at high frequency, followed by a period of sporadic SE+ egg production. These naturally infected flock data support the possibility that the frequency of hens producing SE+ eggs will be increased during specific times.

Data Analysis for Estimating the Percentage of SE-positive Eggs by Transovarian Contamination

Data from Bichler et al.[5] were used to estimate that 8.62% of eggs at lay will be SE-positive from transovarian contamination. These data were collected up to 8 weeks post-inoculation of hens and include contamination in the albumen, yolk and the inner shell membranes. In the first week,

[d] The hen cloaca is located beneath the vagina and above the vent (an opening that serves for egg laying and excretion). This organ is where the reproductive system joins the digestive system.

a relatively high percentage (52%) of contaminated eggs was observed. The uniformity assumption implicitly made is that at any time, 1/8 of the infected hens (over an 8 week period) will be recently infected and laying (potentially) a high percentage of contaminated eggs. At the same time, this assumption suggests the other $7/8^{th}$ of the hens will not be laying a larger percentage of eggs (4.1%). Because the percentage of positive eggs was not decreasing for the later 7 weeks, thus it is not possible to guess or extrapolate the time when the percentage of contaminated eggs would be negligible. For modeling purposes, 8.62% (based on 54 positive results from 592 eggs tested) is assumed. Uncertainty of this percentage is determined assuming that these results were generated from a trinomial distribution, albumen, yolk and inner shell membrane, with $n = 592$.

Molting

After estimating the percentage of transovarian-infected SE-positive eggs from SE-positive non-molted hens, the percentage of SE-positive eggs from SE-positive molted hens by transovarian contamination was estimated. Forced molting is believed to increase the frequency of SE-positive eggs produced by an SE-infected flock. As this is a common practice, molted flocks might produce an increased risk to the consumer. To account for this, weekly molting factors were determined and applied to the percentage of SE-positive eggs produced per week from molted flocks for 10 weeks. A discussion of the effect of molting on hens and role of the immune system in molting is given to provide an understanding of how eggs might be more frequently contaminated by molted hens. This is followed by application of these data to modeling the effect of molting on the percentage of SE-positive eggs produced by an SE-positive flock.

Increased SE egg contamination by molted hens

As laying hens age egg production and quality decreases. Industry producers impose a forced molt on hens that results in increased egg productivity and decreased hen mortality compared with non-molted hens of the same age. Though there are many ways to experimentally induce molting, feed and water withdrawal including light manipulation and special molting diets are typically used. Though molting rejuvenates egg production rates and quality, experimentally and naturally infected hen studies suggest molted hens are more susceptible to SE infection and produce more SE-positive eggs post-molt (Table B8). As molted hens represent a substantial portion of the egg-producing hens, this risk assessment has considered the effect of molting on the production of SE-positive eggs by transovarian contamination.

TABLE B8 EVIDENCE FOR INCREASED SE-POSITIVE EGGS BY MOLTED HENS.

Publication	Study type	% SE-positive eggs by non-molted hen	% SE-positive eggs by molted hen
Holt and Porter[15]	Experimental oral inoculation	0 (0/13)	18 (2/11)
	Experimental oral inoculation	0 (0/105)	2 (3/153)
Holt and Porter[14]	Contact exposed to inoculated hens	0 (0/53)	1.6 (2/124)
Schlosser et al.[3]	Naturally infected	0.02 (14/67000)	0.05 (39/74000)

SE infection susceptibility of molted hens

Molted hens are more susceptible to SE intestinal colonization than their non-molted counterparts are, as evidenced by oral inoculation studies with varying level of SE.[14;15] These data suggest molted hens are more likely to disseminate SE into their environment. Molted hens are also more susceptible to SE infection by contact exposure to experimentally infected hens and can be infected by aerosol transmission.[15] This suggests transmission of SE among molted hens would be more rapid than among non-molted birds, which implies increased SE-positive egg production by molted hens could be due in part to greater within-flock prevalence.

Histopathology of molted hens

Histopathology of infected tissue from molted hens was more severe compared with tissue from non-molted hens. Histological examination of the gastrointestinal tracts of molted SE-infected hens revealed more frequent and severe epithelial cells inflammation of the colon and ceca compared with non-molted SE-infected hens,[15;37] which could allow more frequent access of SE to extra-intestinal tissues, such as the ovary and oviduct.

Cellular immunity of molted hens

To study the relationship between the immune system and molting, researchers investigated varying aspects of the hen immune system. A series of 1992 papers published by the USDA Agricultural Research Services (ARS) suggest the cell-mediated branch of the immune system might be impaired in molted hens. This part of the immune system is critical in activating type 2 thymus dependent B-cells to produce antibodies, stimulating macrophage mediated destruction of extracellular and intracellular pathogens, and activating cytotoxic CD8+ T-cell mediated intracellular pathogen destruction. Holt[38] reported a statistically significant decrease in the numbers of a critical set of T-cells in the serum, CD4+ T-cells, 3 days after feed removal; but serum CD8+ T-cells were not different from controls. CD4+ T-cells are a central part of cellular immunity suggesting that this branch of the immune system of molting hens is impaired.

Holt[13] and Holt and Porter[15] demonstrated the delayed type hypersensitivity (DTH) response was depressed in molted hens 3 and 7 days post-feed removal. This immunological reaction is mediated by CD4+ T_H1 T-cells. CD4+ T-cells differentiate into T_H1 and T_H2 T-cell subtypes upon antigenic stimulation. Differentiation into T_H1 cell subtype results in macrophage stimulation and recruitment to the site of infection as well as B-cell stimulation. Differentiation into T_H2 cell subtype results in a B-cell dominated antibody response. The results of the DTH experiment suggest that T_H1 cells are depressed in molted hens; however, this does not negate a role for T_H2 cells. T_H1 cells are involved in controlling bacterial intracellular infections; thus, molting hens might be more susceptible to infection due to this attenuated immune compartment.

Salmonella spp. are capable of growing within the vesicles of macrophages. These intracellular pathogens survive because the vesicles they occupy do not fuse with the macrophage lysosome, a vesicle containing antimicrobial agents. T_H1 cells can activate the macrophage to induce vesicle and lysosome fusion, thereby increasing the likelihood of pathogen killing. At the same time, the macrophage activates other antimicrobial mechanisms and the $T_H1$cell release cytokines that attract more immune cells to the infection site. The role of T_H1 cells in mediation of intracellular bacteria suggests the increased susceptibility and pathology associated with SE infection in molting hens might be a direct consequence of depressed T_H1 numbers or function during the molting process. However, though T_H1 cells are involved in

generating an antibody mediated response, T_H2 cells are the major helper cells responsible for antibody production. Thus, molting might not greatly affect the serum antibody response to SE.

Summary of Molting and the Hen Immune Response

Molted hens generally produce a higher frequency of SE-positive eggs than do non-molted hens. Molted hens are more susceptible to SE infection by contact exposure and experimental inoculation than non-molted hens. Molted hens in production are likely more susceptible to SE infection and re-infection. Therefore, the percentage of SE-positive eggs produced by SE-infected molted hens by transovarian contamination is increased in the risk assessment.

DATA ANALYSIS OF MOLTING

Percentage of Annual Molted Flocks

To identify the percentage of annual percentage of molted hens in the U.S., this risk assessment used data reported in the 1998 FSIS SE risk assessment.[4] The percentage of flocks that are molted was assumed 22%. The definition of molted hens as determined by USDA-NASS is unclear. Therefore, the period hens will be considered molted is 10 weeks. After 10 weeks, hens will no longer be considered molted for the purposes of determining risk. Using the uniformity distribution assumption, it is assumed that 10% of molted flocks will produce SE-positive eggs for each of the 10 weeks, i.e. 2.2% of all flocks will be molted and considered to be producing a greater frequency of SE-positive eggs each week for 10 weeks.

Effects of molting flocks on percentage of SE-contaminated eggs

To determine the increase of SE-contaminated eggs associated with molted flocks, data from the Pennsylvania SE pilot project were used.[3] Molted hens produced more SE-positive eggs than non-molted hens. The percentage of SE-positive eggs was greater for 10 weeks post-molt and was negligible from 10 to 20 weeks. In this risk assessment, a variable molting factor was applied weekly for 10 weeks to a recently molted flock (22% of flocks).

TABLE B9 DATA USED TO DETERMINE MOLTING EFFECT ON PERCENT SE-POSITIVE EGGS.[3]

Molt Type	Range of Weeks	No. Flocks	No. Eggs Tested	SE-positive
Pre-	-20 to -16	3	7,000	4
Pre-	-15 to -11	9	16,000	1
Pre-	-10 to -6	12	23,000	4
Pre-	-5 to 0	12	21,000	5
Post-	0 to 5	6	9,000	13
Post-	6 to 10	8	19,000	13
Post-	11 to 15	9	18,000	2
Post	16 to 20	10	28,000	11

Let $p(t)$ be the percentage of SE-contaminated eggs, as a function of time. There does not appear to be a clear pattern of the percent SE-positive eggs as a function of weeks before molting. Consequently, for the purposes of modeling, it is assumed that $p(t) = p(0)$ for $t < 0$. Various functions can be used to describe $p(t)$; a desirable function would be one that asymptotically approaches $p(0)$ as t 6 4 and, for small t, is not "too" large. A function that fits this description is:

$$f(t) = \frac{e^{b+ct}}{1+e^{b+ct}} + a \qquad \text{(B5)}$$

for $t > 0$, where a, b, and c <0 are parameters, whose values are to be estimated from the data in Table B9. The parameter a is an estimate of $p(0)$ so that $f(0)$ is set equal to a. Nonlinear regression was performed using the number of positive eggs as the dependent variable, assumed to be distributed as a binomial distribution with parameter n and $f(t)$, where n is the number of eggs tested. The independent variable is the average of the two times defining the range, given in Table B9. Regressions also were performed using related functions, such as using $\ln(t)$ instead of t in Equation B5, or assuming $f(t) = ag(t)$ where $g(t)$ is a function; but the loglikelihood was slightly greater for the function described by Equation B5 and the ratio of $p(t)/p(0)$ was generally the smallest from among those derived from other functions considered. The estimated values of the parameters, standard errors, and correlation matrix are given in Table B10.

TABLE B10 ESTIMATES OF VALUES OF PARAMETERS DEFINED IN EQUATION B1, STANDARD ERRORS AND CORRELATION MATRIX, ESTIMATED WITH 5 DEGREES OF FREEDOM.

	a	***b***	***c***
Estimate	0.000226	-6.0987	-0.2302
Standard Error	0.000054	0.4843	0.0953
a	1.0000	0.2255	-0.4824
b	0.2255	1.0000	-0.8192
c	-0.4824	-0.8192	1.0000

Figure B2 is a graph of the logarithms of the observed percentages and the percentages predicted using Equation B1 and of the results from the nonlinear regression versus logarithm of the number of weeks post-molt (where ln(0) is assigned a value of -2). Figure B3 presents the predicted ratios, $p(t)/p(0)$, of the percentages of SE-contaminated eggs for molted versus non-molted flocks versus the number of weeks post-molt.

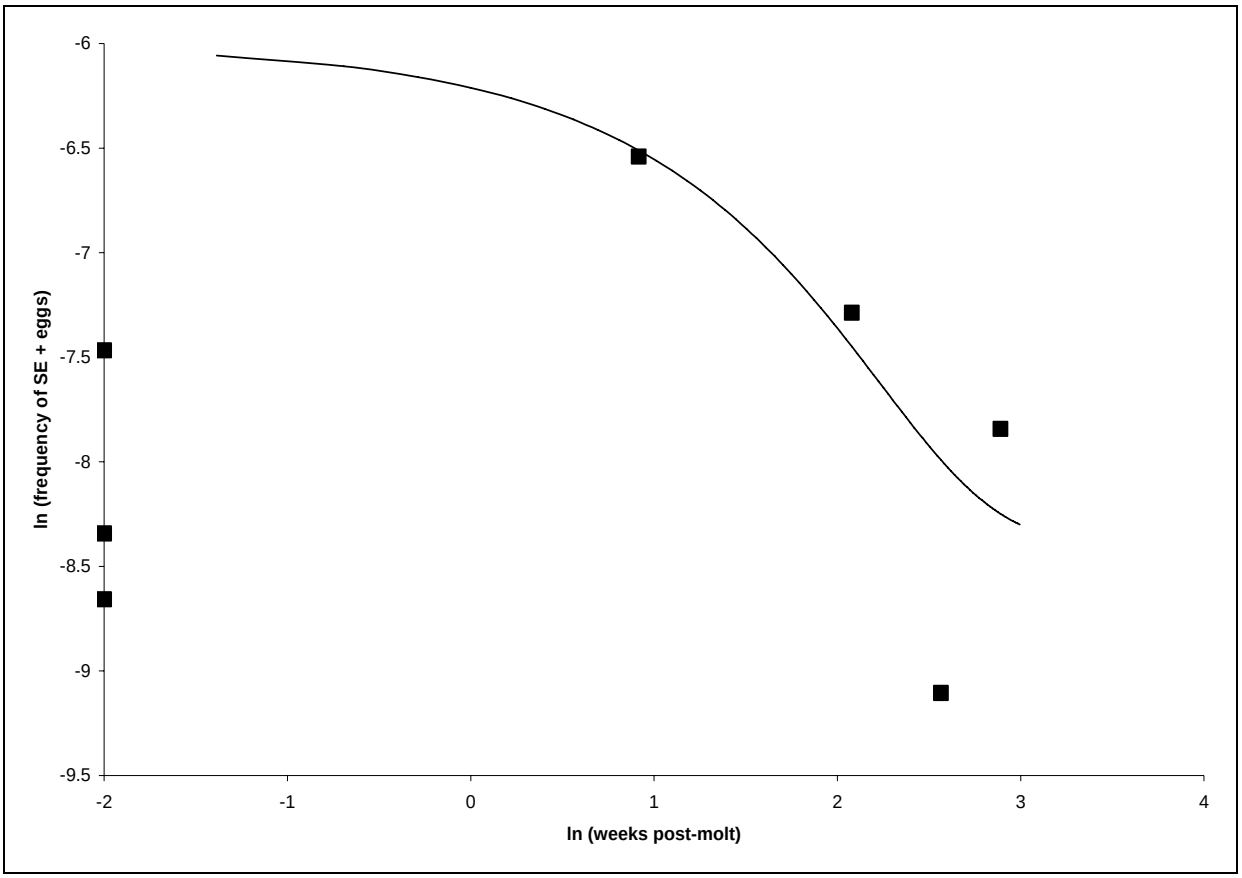

Figure B2 Logarithms of the observed and the predicted percentages predicted versus the natural logarithm of the number of weeks post-molt (where ln(0) is assigned a value of -2).

Molting Factors

For just recently molted flocks, the percentage of SE-positive eggs increases by a factor of about 10 for the first week. This factor decreases weekly and is not considered past 10 weeks for purposes of determining risk. Each weekly molting factor as determined by Figure B3 was not applied uniformly to the 8.62% average of SE-positive egg produced by SE-infected hens over 8 weeks.[5] As mentioned in "Estimating the percentage of SE-positive eggs by transovarian contamination" (above), 52% of SE-positive eggs were produced during the first week of infection, followed by an average of 4.1% for the next 7 weeks of infection. The data are reprinted in Table B11.

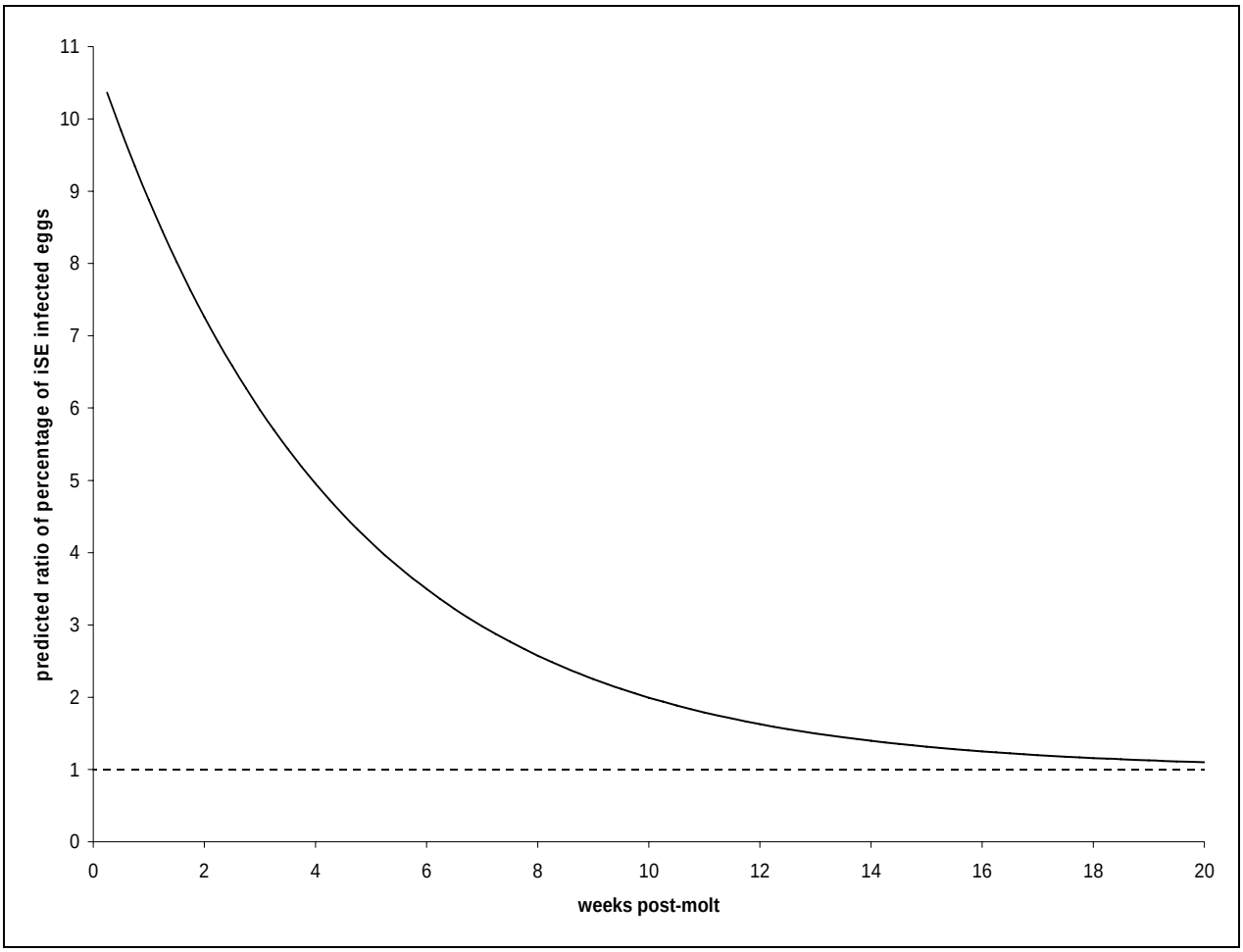

FIGURE B3 PREDICTED RATIOS OF THE PERCENTAGES OF SE-CONTAMINATED EGGS FOR THE RATIO OF MOLTED AND NON-MOLTED FLOCKS VERSUS THE NUMBER OF WEEKS POST-MOLT.

The above weekly molting factors as determined by Figure B3 were applied to the weekly percentages in Table B11. For example, 4.9% SE-positive eggs were laid during week 2 of infection, corresponding to a molting factor of ca. 7.5 (Figure B3); therefore, 37% of the eggs produced by molted hens will be SE-positive during the second week post-molt and the second week of infection. In addition, hens that are in their fourth week of infection and producing 2.4% SE-positive eggs, for example, and 1 week post-molt (molting factor of 10), were considered to produce 24% SE+ eggs (2.4 x 10).

TABLE B11 ESTIMATING THE PERCENTAGE OF SE-POSITIVE EGGS.[5]

	Week [a] 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Total
Total (%) SE-positive eggs	32 (52)	3 (4.9)	3 (3.7)	2 (2.4)	5 (6.0)	2 (2.3)	4 (4.9)	3 (6.0)	54 (8.6)

[a]Weeks post-inoculation.

Because there cannot be more than 100% SE-positive eggs of the eggs produced by any one molted hen, the factor of 10 cannot be directly applied to the percentage SE-positive eggs

produced by SE-positive hens during the first week of infection. Therefore, 100% of the eggs produced by molted hens will be SE-positive during the first week of infection and molt.

Molted hens and egg shell penetration

A molting factor was not applied to the percentage of SE-positive eggs produced by egg shell penetration (*Ep*). We know of no data with which to determine the effect of molting on the prevalence of SE and other *Salmonella* spp. on eggshells.

Fraction of internal egg contamination sites

Transovarian contamination results in deposition of SE within the egg. Depending on where SE was located within the hen reproductive tract, SE could contaminate a range of compartments within the egg. This includes contamination of the yolk (*Ey*), the vitelline membrane (*Ev*), the albumen near the yolk (*Eac*), the albumen far from the yolk (*Eaf*), and the inner shell membranes (*Es*). The growth of SE will differ depending on where the SE is located within the egg. This has a significant impact on the likelihood of outgrowth of SE. For instance, SE deposited in the yolk (*Ey*) or on the vitelline membrane (*Ev*) will have the greatest likelihood and rate of growth compared with SE deposited within the albumen (*Eac* or *Eaf*).

This section presents the model used to compute the percentages of contamination sites within SE-positive eggs, e.g. the frequency of albumen contamination (*Ea*) vs. *Ev* or *Ey* contamination. Table B12 presents a summary of these data from various experimentally inoculated hen studies. The information given includes the SE strain used in the study, hen breed, the route of hen inoculation, the properties and the numbers of eggs analyzed, the numbers of SE-positive eggs and the numbers of contaminations detected in the albumen and the yolk. Unless stated otherwise, the numbers for the latter group are assumed to represent either vitelline membrane (*Ev*) or internal yolk contents (*Ey*) contaminations, or both.[e] *Ey* contaminations are indicated only when the authors explicitly state the interior of the yolk was being sampled.

Fraction of Ey *or* Ev *eggs*

Table B12 presents evidence to support preliminary thoughts about the possible differences in contamination rates among contamination site profiles and possible causes of these differences. Several of these studies did not distinguish between contamination of the vitelline membrane and internal yolk contents. Methodologically, the yolk and the vitelline membrane were cultured for SE together. Consequently, these studies were not useful in identifying the percentage of internal yolk contamination (*Ey*) eggs. To determine this percentage, we used studies by Gast and Holt[33] and Shivaprasad et al.,[39] studies that explicitly reported contamination of internal yolk contents. The eleven *Ey* contaminations reported by Shivaprasad et al.[39] is substantially larger than the three recorded by Gast and Holt,[33] even after taking into consideration the number of samples and different time frames post-inoculation at which the samples were analyzed.

[e] Studies reporting SE yolk infection typically did not distinguish between contamination of the vitelline membrane (*Ev*) or contamination of the internal yolk content (*Ey*).

TABLE B12 PROFILE OF INTERNAL EGG CONTAMINATION SITES.

Strain	Hen Breed	# Eggs Analyzed		Egg Age	Fraction Positive (%)	# *Ea*	# *Ey* or # *Ev*
SE6 PT13a[21]	SPF White leghorn	Oral[a]	623	Collected daily[h], held 4 d, 25°C	NR[b]	151	141
SE6 PT13a[40]			138	Collected daily (4-14 d PI), held 7 d, 25°C	22/138 (16)	NR	NR
SE6 PT13a[41]			874	collected (6-17 d PI) and analyzed daily	25/874 (2.9)	4	21
PT13a[33]			675	collected (4-22 d PI) and analyzed daily[f]	NR	NR	29 3 *Ey* only
Y-8P2[39]	Commercial White leghorn	IC[d]	231	Egg collection unstated, but assumed daily. Eggs stored 2-5 d, 4°C[g]	5/231 (2.2)	4	1 *Ey* only
		IV[c]	274		10/274 (3.5)	8	2 *Ey* only
		Oral	221		6/221 (2.7)	6	0 *Ey* only
27A[39]		Oral	314		17/314 (5.4)	6	11 *Ey* only
Bichler et al.[5]		Oral	592	collected (1-56 d PI) and analyzed daily	44/592 (7.43)	29	40
Okamura et al.[32]	White leghorn Julia	IV	43	collected (1-7 d PI) and analyzed daily	4/43 (9.3)	1	3
Humphrey et al.[28]	Naturally infected 12 free-range hens	NA	451	Collected daily. Stored 20°C, time unknown	5/451 (1.1)[e]	1	3
	Naturally infected 23 free-range hens		68		2/68 (2.94)	1	1

[a]Route of SE inoculation
[b]NR, not reported
[c]IV, intravenously
[d]IC, intracloacally
[e]Egg contents homogenized in 1 sample, unable to determine original location of SE within egg.
[f]Egg collected daily on weekdays and stored 1-2 d at 7.2°C for weekend.
[g]The 314 eggs were collected 1-11 d post-inoculation (PI), constituting all found positive eggs; the study was continued up to 42 d PI, yet no positive findings in the remaining 550 eggs.
[h]Eggs collected on days 1-12, 14, 16, 18, 23 30 and 37 post-inoculation (PI).

The differences between these two studies may be due to sample handling, hen inoculation dose, hen age, hen type, analytical methodologies, and/or SE strain. These issues are important to resolve, as the percentages of *Ey* contamination from these two studies were quite different. Both study protocols were designed to sample yolk contents for estimation of *Ey* contaminations, while minimizing potential mixing of yolk samples with albumen or vitelline membrane. As the relative risk of yolk contamination for each study would be quite different, using both studies would generate a large amount of uncertainty. Therefore, we attempted to resolve these two studies by an analysis of the factors listed above. Each issue is discussed in turn below.

Sample handling

Older eggs are more prone to growth of SE due to the potential for yolk membrane breakdown. Time also allows contaminating SE to migrate into other egg compartments. Therefore, eggs that are not quickly collected after lay and examined for where SE was deposited may not be indicative of the initial site of contamination.

Consider the data from Shivaprasad et al.[39] above. The collection schedule is unclear, so a daily collection was assumed. If the eggs were not collected daily and allowed to remain at room temperature for an undisclosed amount of time prior to being placed at 4^{o}C, then the higher *Ey* events observed in this study could be attributed to SE migration from albumen to yolk. Shivaprasad et al.[39] report the number of days post-inoculation the sample was cultured, suggesting that for this number to be meaningful, the age of the egg was known. It can be inferred from this that the hen egg depositories were checked daily and the eggs collected daily. It can also be reasonably assumed that storage at 4^{o}C would minimize migration of SE from the albumen to yolk contents. These assumptions imply the sampling protocol of Shivaprasad et al.[39] is similar to that of Gast and Holt[33] with respect to their effect on the likelihood of finding yolk positive samples. Building on this interpretation, the differences between data from Gast and Holt[33] and Shivaprasad et al.[39] need to be explained by considering other factors.

Hen inoculation dose

Gast and Holt[33] administered 9 $\log_{10}$ cfu/hen, while Shivaprasad et al.[39] administered 6 $\log_{10}$ cfu/hen for strain 27A. Based on the discussion of a leveling-off effect of doses between 6 $\log_{10}$ and 9 $\log_{10}$ found in Attachment B1, the difference in inoculum dose would not by itself explain the difference between *Ey* contamination frequencies of the two studies.

Hen age

The SE PT13a hens inoculated by Gast and Holt[33] were 6-7 months old compared to 9 and 24 month old hens for SE strains 27A and Y-8P2 inoculated by Shivaprasad et al.[39] It is unclear what effect hen age would have on internal egg contamination by transovarian contamination.[12] The age difference of 6-7 months and 9 months is not likely a factor in the observed differences of *Ey* contaminated eggs. Within the Shivaprasad et al. study,[39] hen age differences might have had an affect on the positional differences observed between strain 27A and Y-8P2 (Table B12).

Hen type

Another difference between studies was that Gast and Holt[33] used specific pathogen-free (SPF) hens and Shivaprasad et al.[39] used commercial hens of the same breed. However, the effect of SPF hens compared with commercial hen of the same breed and a similar age is difficult to interpret and does not provide a plausible explanation for the observed differences in *Ey* contamination.

Analytical methodologies

Another possible explanation for the differences could be the different analytical methodologies used for these studies. Gast and Holt[33] removed internal yolk contents free of contamination from the vitelline membrane (*Ev*) or any adhering albumen (*Eac*) by searing the yolk surface before inserting a syringe to remove the yolk contents. This method likely killed any SE contaminating the surface from *Ev* and adhering albumen (*Eac*) contaminations. Shivaprasad et al.[39] did not use a searing step. Instead, a pair of scissors was used to cut the membrane before the contents were extracted. It is possible this method could have allowed yolk content samples to be contaminated with SE from the vitelline membrane and/or adhering albumen. Cross contamination into the yolk could explain the high yolk contamination (*Ey*) results of the study.

SE strain

The results from Shivaprasad et al.[39] suggest the strain of SE influences the ratio of the numbers of *Ey* or *Ev* to *Ea* contaminated eggs. The authors removed 1 ml of yolk or albumen contents separately, excluding the vitelline membrane. The techniques used could have resulted in cross contamination from the albumen and the vitelline membrane. If cross-contamination for the albumen occurred, then the results cannot be interpreted as *Ev* or *Ey* contaminations and cannot be directly compared to the results of Gast and Holt.[33] In the case of cross-contamination, the observed difference between the two strains (for the strain Y-8P2 there were 18 *Ea*-contaminated eggs versus only 3 *Ey* ones, whereas, for the strain 27A there were 6 *Ea*- versus 11 *Ey*-contaminated eggs (Table B12)), could be differences of *Eac* contaminations as well. However, this difference could be due to the ages of the birds used (9 vs. 24 months) and the differences in the doses (6 $\log_{10}$ vs. 4 $\log_{10}$). Though the differences in this study could be attributed to strain difference, the confounding factors as discussed above make the reasons for this difference difficult to interpret and compare between studies. How these data are to be treated concerning estimating the percentage of eggs that are *Ey or Ev* is discussed below. For the purposes of this risk assessment, data from Gast and Holt[33] were used to determine the fraction of *Ey* or *Ev* contaminated eggs.

*SE inner shell membrane contamination (*Es*)*

SE can contaminate the isthmus and the uterus of the hen oviduct. During egg formation, the isthmus deposits two inner shell membranes onto the outermost albumen and the uterus is responsible for deposition of the outer shell (OS) and the cuticle. Therefore, it is possible SE contaminates the inner shell (IS) membranes due to its presence in the isthmus or prior to the complete deposition of the OS, a process that typically takes 20 hrs to complete.

Contamination of the IS membranes has been explored by Bichler et al.,[5] who found the IS membranes were frequently contaminated when other egg components were also contaminated. Some eggs were found that only had contamination of the IS over the 8-week period (1.7% (10/592) of eggs laid by SE-infected hens were IS positive compared with 7.43% (44/592) yolk- and albumen-positive eggs). These *Es*-only contamination events suggest these were not penetration events from the OS or contaminating albumen, but rather contamination by vertical transmission from the infected isthmus or uterus. Additionally, three studies support the notion

of *Es* contamination (see textbox) and taken together, suggest IS contamination can range between 1.7-15% of SE-positive eggs. These data suggest vertical contamination of the IS membranes can occur; however, it is possible that IS+ results could be due to contamination of other egg compartments. This is the reason the risk assessment focused on eggs that were negative for OS, albumen, and yolk contamination. There will be a small percentage of false IS-positives due to false-negative results of OS, albumen, and yolk contamination due to cross-contamination during sampling. Hence, the *Es* positive frequency is likely to be slightly less than predicted by these studies.

It is unclear how contamination of IS membranes affects subsequent growth of SE. The IS outer and inner membranes are approximately 60 and 20 μm thick, respectively.[42] The outer membrane is relatively porous, but the inner membrane is composed of a fine fibrous matrix of proteins with few pores and is thus likely to present a physical barrier to SE penetration into albumen and migration to yolk. *Es* penetration into albumen, an *Eaf* contamination, seems likely to be time-dependent.

β-N-acetylglucosaminidase activity is particularly active in the IS shell membranes. This enzyme is known to inhibit the growth of Gram-negative bacteria; however, activity is lost rapidly as the egg ages and local pH increased.[44] Therefore, growth of *Es* might initially be inhibited, but could increase as the egg ages. These data together suggest SE in *Es* contaminations are less likely to grow compared to those in *Eaf*, *Eac*, *Ev*, and *Ey* contaminations. At the same time, SE from *Es* contaminations could penetrate IS membranes and become an *Eaf* contamination event. No data are available for prediction of IS penetration. We cannot reasonably predict the frequency or magnitude of transfer from *Es* to *Eaf* contaminations.

Data supporting the hypothesis of vertical *Es* egg contamination

Three additional studies support the hypothesis of *Es* contamination: (i) Miyamoto et al.[43] found hens intravaginally (IVg) inoculated with SE yielded 20% (5/20) SE-positive eggs. Three were OS+, 3 were IS+ and 1 was positive for inner contents. Though these authors did not distinguish which eggs had multiple contamination sites, the data imply 1 egg must have been IS+ only (5.0%) and 2 eggs may have been IS+ only. Seventeen percent (1/6) hens were uterus-positive for SE following IVg inoculation with 7 $\log_{10}$ CFU, suggesting contamination from the uterus could have been the source of the IS contaminated egg. (ii) Okamura et al.[35] reported hens inoculated with 6.7 $\log_{10}$ CFU IVg produced 27.6% (11/40) SE-positive eggs. Two were OS+, 10 IS+ and 3 inner contents-positive. These data suggest 6 were IS+ only (15.0%). (iii) Okamura et al.[32] found hens inoculated with 6.7 $\log_{10}$ CFU intravenously (IV) produced 9.3% (4/43) SE-positive eggs. Two were OS+, 1 IS+ and 4 inner contents positive. These data suggest IS contamination can range between 1.7-15% of SE-positive eggs when no other egg components are SE contaminated. However, contamination rates depend on the route of contamination, with IVg inoculation realizing higher *Es* contamination than oral or IV inoculation.

Es *contamination estimate*

For the purposes of modeling *Es* events**,** it was assumed that the percentage of *Es*-only contaminated eggs among all contaminated eggs is equal to 10/(44+10) = 18.5%. It was also assumed that there is no SE growth within this egg compartment until YMB. Uncertainty of this percentage was determined assuming the numbers of *Es*-only and other contaminations are distributed as a binomial distribution with total number of samples equal to 592.

Data analysis for estimating the fraction of internal egg contamination sites

The percentage of *Es*-only eggs is given in the above analysis. The percentage of *Ey* and *Ev* eggs was calculated using data that estimates the percentage of *Ey* contamination and total SE-positive contaminations. The percentage of *Ea* is determined to be the residual incidents, from which the percentage of *Eac* and *Eaf* is assumed as a state of knowledge variable. The method by which the contaminated eggs were attributed to the different contamination sites is described in Table B13.

Estimating the percentage of yolk (*Ey*) or vitelline membrane (*Ev*) contaminated eggs

Data are not yet available to support an empirically based estimate of the distribution of *Ey* or *Ev* contamination incidents. Instead, data from Gast and Beard[40] and Gast and Holt[33;45] were used to generate subjective probability estimates of these distributions.[f] It was assumed that the similar protocols used in these studies would produce similar percentages of contaminated eggs.

To estimate the percentage of *Ey* or *Ev* eggs the percentage of total contaminated eggs and of *Ey* or *Ev* contaminations is needed. With these two numbers, the percentage of *Ey* or *Ev* eggs of all SE-positive eggs can be calculated. To determine the percentage of total eggs contaminated, it can be assumed that approximately 16% (22/138) of eggs laid were infected.[40] To account for a false-negative rate due to difficulties in recovering SE by culturing, it can be assumed that 20% of the eggs were actually infected. To determine the percentage of *Ey* or *Ev* eggs, it can be assumed that approximately 2.4% (21/874) were *Ey*- or *Ev*-contaminated.[45] To account for a false negative, it can be assumed that 6.37% of the eggs were *Ey*- or *Ev*-contaminated.

To calculate the percentage of *Ey* or *Ev* eggs of all SE-positive eggs, 6.37/20 = 32% of the eggs could be *Ey*- or *Ev*-contaminated. However, based on the discussion above (see Fractions of *Ey* or *Ev* eggs), the effect of strain on this percentage is unclear. Therefore, the percentage of contaminated eggs that are *Ev*- or *Ey*-contaminated eggs was assumed a state of knowledge variable ranging from 1% to 50%.

Estimating the percentage of yolk (*Ey*) contaminated eggs

Gast and Holt[33] reported 4.3% (29/675) *Ey* or *Ev* eggs; of these 29 eggs, 10.34% (3/29) were *Ey* eggs. Therefore, 10.34% of eggs are estimated to be *Ey*-contaminated. This percentage is assumed constant for this risk assessment, varying only due the uncertainty of the estimated ratio, *R*, which is based on a function of two random variables, n_y and n_v, where n_y is the number of *Ey* contaminated eggs and n_v is the number of *Ev* contaminated eggs (assumed not infected in the yolk). *R* is equal to $n_y/(n_y+n_v)$, where n_y and n_v are assumed to be distributed as a binomial distribution with probability parameters equal to 3/675 and 26/675 corresponding respectively to n_y and n_v and number parameter equal to 675.

Estimating the percentage of albumen contaminated (*Ea*) eggs

The above analysis provides an estimate of 1-50% for *Ev* and *Ey* contaminations for *Ev*, *Ey* and *Ea* eggs. By subtraction, the percentage of *Ea* eggs from the total population of SE-positive eggs is 99% (100-1) to 50% (100-50).

[f] We are cognizant of the possible implications of the data from Shivaprasad et al.[39] in calculating the fraction of *Ey* eggs, but do not use these data explicitly. Results of these experiments should be reproduced prior to being used in a risk assessment.

Estimating the percentage of albumen contaminated near (*Eac*) or far (*Eaf*) eggs

The remaining parameter to be determined is the percentage of *Eac* contaminations from among *Ea* contaminations that are not also *Ey*, *Ev* or *Es* contaminations. An *Eac* contamination can be caused by migration of an *Eaf* contamination from the oviduct. It can also occur by deposition of albumen onto the yolk in the SE-infected upper magnum of the oviduct, though the opportunity for this to happen, given that the yolk and the vitelline membrane are not contaminated, is limited: the yolk travels down the magnum, albumen is spooled over the vitelline membrane; as the albumen that could harbor *Eac* contamination will be a smaller proportion of the total albumen, *Eac* contaminations will constitute a lower fraction of *Ea* contaminations, given the yolk and vitelline membrane are not contaminated. As the transit time for the yolk in the magnum is approximately 3 hours, the majority of this time in the oviduct will likely result in *Eaf* and not *Eac* contaminations. *Eaf* contamination can occur prior to the deposition of the inner shell membrane from the isthmus, as here the egg transit time is approximately 1 hour.

Eaf contaminations are expected to constitute a greater proportion of total *Ea* contaminations unless the magnum is preferentially infected by SE, which could occur for particular SE strains. As a lower bound, we assumed as little as 20% of the *Ea* contaminations are *Eac*, based on the belief that *Eac* compartment volume constitutes at least this percentage of total egg albumen volume. The percentage of *Eac* contaminations from among *Ea* contaminations was assumed a state of knowledge variable ranging from 20 to 50%.

TABLE B13 PERCENTAGES OF CONTAMINATION SITES.

Contamination Site	Estimate (%)	Source
Es	18.5 of all SE+ eggs	Bichler et al.[5]
Ey or *Ev*	1 to 50 of *Ea*, *Ey* or *Ev* SE+ eggs	State of knowledge variable
Ey	10.35 of *Ey* or *Ev* SE+ eggs	Gast and Holt[33]
Ev	89.65 of *Ey* or *Ev* SE+ eggs	100-*Ey*
Ea	99 to 50 of *Ea*, *Ey* or *Ev* SE+ eggs	100-(*Ey* or *Ev*)
Eac	20-50 of *Ea* SE+ eggs	State of knowledge variable
Eaf	80-50 of *Ea* SE+ eggs	100-*Eac*

Percentage of SE-positive eggs by egg shell penetration

Spent hen surveys were used to estimate the percentage of *Salmonella* spp.-positive flocks and within-flock prevalence and results from controlled experiments were used to estimate the percentages of surface SE-positive eggs and shell penetration events.

Mechanisms of shell contamination and egg shell penetration

The process responsible for egg shell contamination by infected birds is not clear. Shell contamination most likely depends on both intestinal and oviduct infection. The egg surface can be contaminated with feces containing *Salmonella* during expulsion of the egg from the hen. The egg surface can also be contaminated within the hen reproductive system after formation of the shell. Both routes lead to contamination of the egg surface and, potentially, inner eggs contents. Gast and Beard[21] identified a correlation with SE fecal contamination and egg shell contamination, suggesting colonization of the intestinal tract by SE is important for egg shell

contamination. In addition, Humphrey et al.[12] found shell-positive eggs could be produced by hens that were fecally negative for SE.

Once *Salmonella* is deposited on the surface of an egg, it must overcome several barriers to gain access to the albumen. The shell of the egg is covered by a thin glycoprotein layer known as the cuticle. This structure serves to make the shell resistant to water, plugging the some 6,000-10,000 pores of the egg shell. The cuticle can be unevenly distributed over the egg surface and it can be damaged by washing or desiccation. It is possible SE can be deposited onto the outer shell before deposition of the cuticle, in which case they may then cross through the pores of the outer shell. This action is facilitated by a decrease in external vis-à-vis internal egg temperature. As the external temperature declines, negative pressure is exerted from the egg due to the contraction of the egg air sac. Surface bacteria can then be aspirated through the outer shell and into the egg. To reach the albumen, bacteria would then need to cross the inner shell membranes.

Limitations of data from Schoeni et al.[6]

The data presented by Schoeni et al.[6] suggest SE and other *Salmonella* can penetrate the egg shell. However, limitations of the data must be considered to interpret the results of this study properly. First, sterilized feces were used to contaminate the eggshells and therefore the inoculated SE was the only bacteria present. It is likely that under natural conditions, multiple bacteria types would be present. The presence of these indigenous fecal bacteria would likely alter the ability of SE to survive and penetrate the egg shell. Therefore, these in vitro data might overestimate the frequency of this event as well as the levels of internalized bacteria. Second, eggs used for penetration studies were acclimated to 35^{o}C, inoculated with *Salmonella*, and placed at 4^{o}C. As a greater temperature differential between the environment and the internal egg temperature will likely increase the potential for *Salmonella* to be aspirated into the egg, this study may have overestimated *Ep* (if shell contaminated eggs on a farm are allowed to cool below 35^{o}C before placement at 4^{o}C) or underestimate *Ep* (if shell contaminated eggs on a farm are placed at 4^{o}C before they reach 35^{o}C). Nevertheless, these data do suggest SE can penetrate the egg shell and become deposited within the albumen. Consequently, the data describing *Ep* results from Schoeni et al.[6] were incorporated in the risk assessment.

Frequency of shell contamination

Data from experimentally and naturally infected hens suggests shell eggs can be topically contaminated from eggs produced by SE-infected hens (Table B14). To estimate the percentage of SE surface-positive eggs, data from Bichler et al.[5] were used. This study analyzed eggs within 1 day following lay from young hens orally inoculated with SE. A naturally infected hen study was not used for methodological reasons. Humphrey et al.[46] collected eggs from a farm and stored the eggs at room temperature (20^{o}C) for an unspecified time before transit to a laboratory for microbial examination. It is known that *Salmonella* can rapidly die on egg shells, particularly in low humidity and temperature above 4^{o}C.[47] Moreover, Humphrey et al.[46] investigated SE contamination, but not that by other *Salmonella* spp.; therefore, the data would most likely underestimate the frequency of *Salmonella*-positive shell eggs. These data, taken together, suggest shell contamination will vary over a population of hens.

TABLE B14 FREQUENCY OF SHELL CONTAMINATION.

Publication	Study type	Hen age (weeks)	Inoculation route	% SE Shell+
Gast and Beard[21]	Experimental[a]	27	oral	12 (6/49)
		37		11 (5/42)
		62		53 (8/15)
Shivaprasad et al.[39]		104	oral	1 (2/221)
			IV[b]	2 (5/274)
			IC[c]	5 (12/231)
Bichler et al.[5]		25	oral	34 (201/592)
Humphrey et al.[47]	Natural	NR[d]	NA[d]	1 (21/1952)

[a]Hens experimentally inoculated with SE.
[b]IV, intravenously.
[c]IC, intracloacally.
[d]NR, not reported. NA, not applicable.

Frequency of egg shell penetration

Methods of Schoeni et al.[6]

To investigate shell penetration, sterilized chicken feces were added to shell eggs. Eggs were incubated for 30 minutes at 4, 25, or 35ºC before inoculation of feces with 1 of the three *Salmonella* serotypes at final levels of 4 $\log_{10}$ or 6 $\log_{10}$ cfu/g feces. Each egg was stored for an additional 30 minutes at the initial incubation temperature before storage at 4 or 25ºC. The study design included a test scenario intended to simulate hatchery conditions (incubated at 35ºC for 30 minutes, followed by storage at 4ºC). Eggs were analyzed 1, 3, 7, and 14 days post-inoculation. The 7 and 14 day results were not considered for modeling *Ep* because *Salmonella* shell-contaminated eggs will typically be removed from the farm and washed within 1 week. Only those egg penetration data collected within the first week of lay are relevant to current egg production practices.

We used the work of Schoeni et al.[6] to calculate the percentage of SE shell-contaminated eggs that would be penetrated by SE and other *Salmonella* spp.

Schoeni et al.[6] studied penetration events (*Ep*) for three *Salmonella* serotypes (Enteritidis, Typhimurium, and Heidelberg) through egg shells into egg contents. The patterns of penetration for SE differed from *S.* Typhimurium and *S.* Heidelberg. The data used to identify the percentages of through shell penetration events (*Ep*) are given in Table B15. The percentage of *S.* Typhimurium and *S.* Heidelberg penetrating the shell were combined due to data similarity.

TABLE B15 PERCENT SHELL PENETRATION (EP) BY SALMONELLA SPP.[6]

***Salmonella* spp.**	**1 day**	**3 days**	**Total % shell positives**
S. Enteritidis	37.5% (3/8)	37.5% (3/8)	37.5 (6/16)
S. Typhimurium (ST)	25% (2/8)	12.5% (1/8)	18.8% (3/16)
S. Heidelberg (SH)	37.5% (3/8)	12.5% (1/8)	25% (4/16)
ST + SH	31% (5/16)	12.5% (2/16)	21.9 (7/32)

Other experimental results for treatments of eggs with 4 $\log_{10}$ cfu/g feces were not tabulated but summarized by the authors in their results section.[6] At 25ºC, all *Salmonella* strains grew in feces by 1-2 $\log_{10}$ by day 1 and by 4-5 $\log_{10}$ by day 3. Half of the contents of treated eggs (n = 12) inoculated at 4 $\log_{10}$ cfu/g feces and stored at 25 ºC were positive for unspecified *Salmonella* serotypes by day 3. Two of these egg contents were enumerated: 1.9 $\log_{10}$ cfu/g of SE (ca. 3.7 $\log_{10}$ cfu/egg) and 4 $\log_{10}$ cfu/g *S.* Heidelberg (ca. 5.8 $\log_{10}$ cfu/egg). At 4ºC, SE and *S.* Typhimurium declined in feces, while *S.* Heidelberg increased in feces by 0.3 $\log_{10}$ at day 3. *Salmonella* strains were not detected in contents of eggs stored for 3 days at 4ºC.

Data for estimating the percentage of SE-positive eggs by egg shell penetration

As with estimating the percentage of SE-positive eggs by transovarian contamination, no study exists to estimate this percentage directly for shell penetration. Data from spent hen surveys (Table B16) were used to estimate the percentage of SE-positive eggs by egg shell penetration and the within-flock percentage of *Salmonella* spp.-infected hens. Following these estimates, results from controlled experiments were used to estimate the percentages of surface SE-positive eggs and shell penetration events (*Ep*).

The approach of modeling *Ep* contaminations is similar to the approach that was used for modeling the percentage of SE transovarian-contaminated eggs. However, unlike the latter, we did not have data describing the distribution of the within-flock percentage of hens that are infected with *Salmonella* spp., or data that could be used to estimate the percentage of flocks that are *Salmonella* spp.-infected. The only information available is from spent hen surveys that report a high percentage of flocks that are infected (Table B16).

TABLE B16 PERCENTAGE OF *SALMONELLA* SPP. POSITIVE FLOCKS BY SPENT HEN SURVEYS.

Publication	% *Salmonella* spp. positive flocks
Dreesen et al.[16]	97.4
Ebel et al.[7]	86.0
Waltman et al.[18]	100.0
Hogue et al.[8]	98.0
Average	95.4

Some of these differences might be explained by regional and seasonal effects as well as other environmental factors and experimental methodologies. From these data, it seems reasonable to surmise that greater than 90% of spent hen flocks are *Salmonella* spp.-infected; however, as discussed above (see Susceptibility to SE and competing *Salmonella* spp.), *Salmonella* spp. infection rates for spent hens are likely to overestimate that of commercial hens of laying age. For the risk assessment, we assumed that 95.4% of flocks, based on the average of the 4 spent hen surveys above, are infected with *Salmonella* spp.

For the within-flock percentages of infected hens, the only information regarding the distribution of *Salmonella* spp.-infected hens is given by 2 of the 4 spent hen studies above. Waltman et al.[18] reported that using pooled samples of 3 or 5 ceca, 76% of flocks had isolation rates of 50% or greater and 37% of flocks had isolation rates of 75% or greater. Samples were taken from the southern U.S., and it did not appear that a probability designed survey was used for sample selection. Samples from 81 flocks were examined from nine states. The percentage of all *Salmonella*-positive samples was reported at 65.4% (from 3,700 samples) and the percentages did not differ greatly by state (the largest percentage was 83.3% from a state with 120 samples). Using Equation B1 with an assumed false-negative test rate of 10% and 4 ceca per sample (assumed average value), the percentage of hens infected was determined (Table B17).

TABLE B17 PERCENTAGE OF *SALMONELLA*-POSITIVE HENS ESTIMATES.

SE-positive isolation rate	Estimate of % hen positives[a]
50%	18.4
75%	36.1
65% (total)	27.7

[a]Application of Equation B1 with false-negative rate of 10% and 4 ceca/sample.

It is assumed that p is distributed as a beta distribution, with parameters α and $\exists$. Estimates of values of α and $\exists$ are determined as follows. Let

$$I(x|\forall,\beta) = \int_0^x beta(\,p|\,\alpha,\,\beta)dp \qquad \text{(B6)}$$

be the cumulative distribution of the beta distribution with parameters, $\forall$ and β. The estimated values of $\forall$ and $\exists$ are those that minimized the sum of squares of the three differences: $I(0.184|\forall, \beta)$ - 0.24; $I(0.361|\forall, \beta)$ - 0.63; and mean of the beta, $\forall/(\forall+\beta)$ – 0.277. The derived values are, α = 2.23315 and β = 4.914942, and the mean is 31.5%.

In the study by Dreesen et al.,[16] with 3 ceca pooled per sample, 10.5% of the flocks had isolation rates of 50% or greater,1 flock had 0%, and another flock had 100%. The mean over the 38 flocks was 20.3% and the median was 15%. The samples used in this study were from the southeastern U.S. By using Equation B1, the percentage of infected hens corresponding to the isolation rates of 15% and 50%, is estimated to be 5.9% and 24%, respectively, and, corresponding to the 20.3% percentage of samples that were positive, the percentage of hens positive is estimated to be 8.2% (Table B18).

TABLE B18 ESTIMATES OF THE PERCENTAGE OF *SALMONELLA* SPP. POSITIVE HENS.

SE-positive isolation rate	Estimate of % hen positives[a]
15%	5.9
50%	24.0
20.3% (total)	8.3

[a]Application of Equation B1 with false-negative rate of 10% and 3 ceca/sample.

If it is assumed that the distribution of the within-flock percentage, p, is distributed as a beta distribution, beta($p|\forall, \beta$), then $\forall$ = 0.7230 and β = 7.454, are the values of $\forall$ and β that minimized the sum of squares of the three differences as in the above paragraph. The mean of this beta distribution is 8.8%, which is reasonably close to the overall estimate of 8.2%.

The Waltman et al.[18] and Dreesen et al.[16] studies represent flocks from the southern U.S. Waltman et al.[18] comments that *Salmonella* were detected from every flock, and surmise the high rate of isolation "may be a consequence of the use of a more sensitive and selection isolation method than previously used." Therefore, isolation methods of *Salmonella* spp. by Waltman et al.[18] were more comprehensive than that of Dreesen et al.[16] (see false-negative rate of spent hen survey). Consequently, the results from Waltman et al.[18] were used for determining the distribution of the within-flock percentage of hens that are infected with *Salmonella* spp. A

further reason to concentrate on this data is the realization that other regions of the U.S. would have higher prevalence of *Salmonella*, if the same relationship seen for SE prevalence holds for *Salmonella* spp.[1] For SE, it is reported that the prevalence for the southern states is lower than that for other states.[16;18] Thus, the distribution of p was assumed to be a beta distribution, with $\alpha = 2.162$ and $\beta = 4.647$.[18]

The distribution reflecting the uncertainties of the estimated values of $\forall$ and β was obtained by bootstrapping. A total of 12,000 simulations were generated, where for each simulation, 81 (representing the 81 flocks that were studied) independent random variables, y, were generated from a beta distribution with parameters $\alpha = 2.23315$ and $\beta = 4.914942$. These were transformed by, $x = 0.9(1-(1-y^4))$, so that the 81 values of x represent the fractions of positive samples for the flocks, assuming that samples consisted of 4 bird ceca and a false-negative rate of 10%. The mean value of y and the percentages of the 81 values of x greater than or equal to 50%, and 75% were determined, and from these three values, values of $\forall$ and β were determined, as described above. Several sets of initial values were used for solving the equations; however, for 2% of the bootstraps, a solution was not obtained, or the solution that was obtained had values of $\forall$ and β very large, greater than 20, or very small, close to 0, and thus were excluded. The square root of the 11,760 generated values of $\forall$ and β that were used were nearly symmetric (skewness coefficients equal to 0.08 and –0.15, respectively), with kurtosis coefficients of 0.22 and 0.51, respectively. The mean of the square root values were 1.50942 and 2.23851, which, when squared, equal 2.2783 and 5.0109, respectively, corresponding to α and $\exists$. The correlation of the square roots of α and $\exists$ is 0.94558. An Edgeworth approximation, using the kurtosis coefficient is used to generate values of parameters of the beta distribution reflecting the uncertainty.

A final step in the calculations needed was the percentage of SE strains from among all *Salmonella* strains infecting hens within a flock assumed not to be SE free. The Barnhart et al.[17] spent hen survey reported 0.9% SE from among the total *Salmonella* isolates found. Allowing for a possible increase in SE prevalence over the last decade, we assumed that 2% of the *Salmonella* strains that have infected a flock are SE. A summary of the assumptions used for modeling *Ep* events is presented below.

Assumptions Used for Modeling

There were six basic assumptions used for the risk assessment modeling.

1) The percentage of flocks, ψ, that have at least 1 hen infected with SE is assumed to be the product of two values, f and g, where $f = 0.096$ and $g = 2.065$ (= 95/46). The uncertainty associated with estimate ψ is accounted for by generating values, f' and g', such that f' is distributed as a lognormal distribution with mean equal to 0.096 and standard deviation equal to 0.052, and $1/g'$ is distributed as a normal distribution with mean equal to $1/g$ and standard deviation equal to $g^{-1}[(g-1)/95]^{1/2}$.

2) For an SE-infected, non-molting flock, the percentage of SE-infected hens, p, is assumed to follow a Weibull distribution, $W(p) = 1- \exp(-(p/c)^b)$, with values of parameters $b = 0.43015$ and $c = 0.005389$. To determine the uncertainty associated with these parameters, values b' and c' are generated by first generating values s' and $:'$ assuming that they are distributed as a bivariate normal distribution with mean

equal to (-ln(b), ln (c)) and standard errors equal to 0.36309 and 0.10775, respectively, with correlation of –0.91281, and then computing $b' = \exp(-s')$ and $c' = \ln(\therefore')$.

3) The percentage of SE-contaminated eggs, q, that an SE-infected hen lays is assumed to be equal to 54/592 (= 8.615%). Therefore, the percentage of eggs that are infected within an infected flock is equal to pq, where p is the percentage of infected hens within an infected flock, as defined in assumption 2. The percentages of contaminations of types *Ey*, *Ev*, *Eac*, *Eaf*, and *Es*, are determined as follows:

   a) The percentage, q_s, of eggs that are *Es* contaminations (that are not *Ea*, *Ev*, or *Ey*-infected) is equal to 10/592. The percentage, q_h, of eggs that are *Ea*, *Ev*, or *Ey*-infected is equal to 44/592. Thus, $q = q_s + q_h$. The uncertainty of these estimates is accounted for by considering the numbers, n_h and n_s, where n_h is the number of *Ea*, *Ev*, or *Ey* contaminations, and n_s is the number of *Es* contaminations that are not *Ea*, *Ev* or *Ey* contaminations, to be distributed as a binomial, with probability parameters, q_h and q_s and number parameter equal to 592.

   b) The percentage, $q_{(v,\,y),}$ of SE *Ea*, *Ev* or *Ey*-contaminated eggs that are *Ev* or *Ey*-contaminated eggs is assumed to be a state of knowledge variable ranging from 1% to 50%.

   c) The percentage, $q_{y|(v,y)}$ of *Ey*-contaminated eggs from among the *Ey* or *Ev*-contaminated eggs is assumed to equal 10.35% (3/29). The uncertainty of this parameter is accounted for by generating random variables, n_y, n_v from a binomial distribution with probability parameters equal to 3/675 and 26/675 corresponding respectively to n_y and n_v, and number parameter equal to 675.

   d) The percentage of *Eac* contaminations among *Ea* contaminations is assumed a state of knowledge variable ranging from 20% to 50%.

4) For a molted flock (up to 20 weeks post-molt), the above percentage of contaminated eggs depends on the weeks post-molt, t. The percentage derived in assumption 3 is multiplied by a factor, $R(t)$, where,

$$R(t) = \frac{e^{b+ct}}{a(1+e^{b+ct})} + 1$$

for $t > 0$, where a, b, and $c < 0$ are parameters determined from Table B4. To determine uncertainty of $R(t)$, values of a', b' and c' are generated, assuming that the standardized values $z_x = (x' - x)/s_x$, where $x = a$, b or c, and s_x represents the standard error of x, are distributed as a trivariate t-distribution with 5 degrees of freedom, with correlation matrix determined from Table B4.

5) The percentage of flocks that are molted is assumed to be 22%.[4]

6) The percentage of eggs that are *Ep*-contaminated is modeled in a similar fashion as that for the percentage of eggs that are SE-contaminated through the transovarian route.

   a) It is assumed that the percentage of flocks that are infected with *Salmonella* spp. is 95% (without accounting for uncertainty).

   b) It is assumed that the within-flock percentage of infected hens, *p*, is distributed as a beta distribution, beta($p|\alpha$ = 2.23315, $\exists$ = 4.914942). Values of α' and $\exists$' reflecting the uncertainty of α and $\exists$ are generated as follows: generated standardized values from a bivariate normal distribution with zero means, unit standard deviations, and correlation of 0.94558, say z_1 and z_2, respectively, are adjusted by computing

$$z_{j'} = z_j + \frac{\kappa_4 (z_j^3 - 3z_j)}{24}, \quad j = 1, 2 \qquad \text{(B7)}$$

   where κ_4 is the kurtosis. For $\alpha^{1/2}$, κ_4 = 0.22 and for $\exists^{1/2}$, κ_4 = 0.51. These adjusted values, $z_{j\mathrm{N}}$ are multiplied by the corresponding standard deviation (0.210 for $\alpha^{1/2}$ and 0.3605 for $\exists^{1/2}$), added to the corresponding mean values (2.23315 for $\alpha^{1/2}$ and 4.914942 for $\exists^{1/2}$), and then squared to calculate the simulated values of α' and $\exists$'.

   c) The percentage, *q*, of shell-contaminated eggs laid by infected hens is assumed to be equal to 201/592 (= 33.95%). The uncertainty is accounted for by generating q' assuming that q' is distributed as a normal distribution with mean equal to *q* and standard deviation is $(q(1\text{-}q)/592)^{0.5}$.

   d) The percentage of shell-contaminated eggs that become *Ep* contaminated depends on the strain of *Salmonella*. If an SE strain, the percentage is 37.5% (6/16); if not SE, the percentage is 21.9% (7/32). The uncertainty of these percentages is accounted for by generating random variables that are normally distributed with mean equal to the percentage, *w*, and standard deviations equal $(w(1\text{-}w)/n))^{0.5}$, where *n* is 16 (for SE) or 32 (for non-SE strain). If the calculations are being performed for flocks assumed to be SE-positive flocks, then it is assumed 2% of the strains within the flock are SE.

Attachment B1: Experimentally inoculated hens and naturally infected hens

The published data present an unclear picture of the percentage of SE-positive eggs produced by infected birds from infected flocks. Numerous confounding factors attributing to variation among data including strain of SE, breed of hen, husbandry practices, and so on. In addition, results from factors inherent in the type of study conducted, e.g. experimentally inoculated or naturally infected hens, might contribute to this variation.

Much of the data presented in this annex were generated from hens experimentally inoculated with SE. These types of studies allow for better control of variables and, as a result, clearer interpretations; however, their representation of naturally infected flocks is unclear. Others studies focus on hens naturally infected with SE. This study type might best represent the typical commercial layer flock; however, this study type is difficult to interpret and many variables such as when the flock was infected, percentage of birds infected and re-infected, and the presence of other *Salmonella* serotypes, etc. are often unknown. Therefore, the data must be interpreted with the knowledge that variation among flocks, hens and eggs is likely to be great. In the following paragraphs, the two study types are compared based on effect of strain on egg contamination; effect of specific pathogen free hens on egg contamination; effect of re-infection on egg contamination; and effect of inoculum size on egg contamination. We discuss the features of experimental and naturally infected hen studies and acknowledge their benefits and limitations.

Effect of SE Strain on Egg Contamination

For experimentally inoculated hen studies, investigators typically use an SE strain associated with human illness or egg contamination. This strain may be used multiple occasions to minimize variability between experiments. Multiple studies have utilized various SE strains to experimentally inoculated hens to determine the frequency of SE-positive eggs produced. This discussion focuses on the seminal work of Gast and colleagues, as we utilized much of their work in completing the risk assessment.

Gast and colleagues often used 1 SE strain (PT 13a, SE6) and 1 hen line (SPF single-comb white leghorn) in their experiments. The SE strain was originally isolated from egg yolk and was selected because, "SE6 was the only one of five *S. enteritidis* strains examined that was associated with the production of a significant number of intact eggs with contaminated yolks following oral inoculation of hens."[21] Though it appears SE6 is capable of increased egg contamination in this hen breed, it is unknown how representative this strain is in the natural SE population in the U.S. SE6 could be representative of at least some SE strains in general, as the virulence mechanisms that afford SE6 more frequent egg contamination could also permit greater dissemination, lengthier hen colonization and/or environmentally out-compete other SE strains. At the same time, SE6 might only produce this phenotype in this particular hen breed. Regardless, it is difficult to estimate the frequency of this particular strain within the commercial hen population and therefore impossible to determine if experimental infection by SE6 would overestimate or underestimate SE-positive egg production in a naturally SE-infected flock.

Effect of Specific Pathogen Free Hens on Egg Contamination

The hen immune response to infection of SE will in part determine the outcome of the infection. For example, a hen unable to mount an immune response might produce more SE-positive eggs and therefore a greater risk. Hens used in experimental inoculation studies may be specific pathogen free (SPF), i.e. hens which have not previously been exposed to *Salmonella*. This is significant as it is possible commercial hens are exposed to different *Salmonella* serotypes over the course of their egg producing life.[4] Different *Salmonella* serotypes can share many surface structures that are immunogenic to varying extents, i.e. create an immune response. Therefore, birds previously exposed to other *Salmonella* spp. would be more likely to mount a quicker immune response based on these shared surface structures. For SPF hens, these birds should be practically naive to *Salmonella* surface structures and might develop a slower immune response than their *Salmonella*-exposed counterparts might. This might suggest SPF hens are relatively more susceptible to SE infection and therefore might produce more SE-positive eggs.

The actual effect of previous exposure to other *Salmonella* serotypes on the protectiveness of SE infection is unclear. Factors, such as surface structures, that allow SE to better colonize reproductive tissues and subsequently contaminate eggs,[32;35;37] are likely absent from the more common *Salmonella* strains harbored by hens. This is supported by research demonstrating that hen immunization with a modified live *S.* Typhimurium strain did not decrease SE-positive egg contamination when challenged with SE.[48] In fact, SE-positive cultures from reproductive tissues, ceca, intestinal tissues, and other viscera were not statistically different between immunized and non-immunized hens. This could be attributed to an overall poor immune response to the vaccine strain in this hen breed; however, levels of anti-*S.* Typhimurium LPS serum antibodies from vaccinated birds were significantly elevated above control birds during challenge by SE. These data suggest prior infection with *Salmonella* might not mitigate SE infection or egg contamination to a significant extent. Alternatively, vaccination with *S.* Typhimurium strain χ3985 (an attenuated strain originally highly virulent as determined by the 1 day old chick virulence model) resulted in no internal egg contamination from hens after challenge with SE strain 27A PT8.[34]

The above data show in some circumstances the protectiveness of previous *Salmonella* challenge to SE infection and egg contamination will be effective, while in another circumstances it may not be; this is likely hen breed and strain dependent. Therefore, it is difficult to predict the impact on the risk assessment of using experimentally infected SPF hen data.

Effect of re-infection on egg contamination

During the course of an infection for a single hen, SE can be shed and expose other hens to SE. This can happen for an experimentally infected group of hens and a naturally infected flock. Hens previously exposed to SE and given a time to mount an immune response, will be less susceptible to re-infection by the same SE strain. However, the ability for experimentally infected hens compared to naturally infected hens to mount an effective response against SE will differ.

For SPF hens previously exposed to SE (non-naïve), re-infection with SE seems unlikely to effect egg production. Re-infection of experimentally inoculated hens could happen during the course of an experiment where birds are housed in the same room through, for instance, contact

or aerosol transmission. SPF hens, under typical infection conditions of 7-9 $\log_{10}$ cfu/hen, produce a strong and quick serum antibody response that is specific for SE.[12;33;39;49-51] Though little is known about the formation of memory immune cells in hens, this type of strong antibody response will likely result in memory cells protective to repeated challenge of SE. Indirect evidence for hen immune memory is provided by immunization studies where a second immunization with the vaccine results in a quicker and more sustained antibody response.[48;52] Therefore, experimentally inoculated SPF hens re-infected with SE by contact or aerosol infection during the course of an experiment will probably not result in re-infection and not affect the frequency of SE-positive egg production following the initial inoculation.

However, this conclusion might be dependent on the strain used in the challenge experiment. SE can undergo natural mutation and change phage type (PT) status. These processes could result in SE strains not well recognized by the hen's memory immune system. However, as an increase in the frequency of SE-positive egg production is not observed beyond 2 weeks post-inoculation under experimental conditions, re-infection unlikely alters SE-positive egg production in experimentally inoculated hens.

In the case of naturally infected flocks, re-infection, and therefore, the state of immune memory, might be important. Naturally infected birds that received a sufficient SE dose to stimulate an adaptive immune response with memory will probably not alter their likelihood to produce SE-positive eggs due to re-infection. However, hens exposed to low levels of SE will probably not produce immune memory cells because of low levels of antigen are likely inadequate to stimulate the memory response. These birds might clear the infection by innate immunity (never developing an adaptive immune response), become contaminated by outgrowth of SE (developing an adaptive immune response with memory), or become chronically colonized at low levels (no adaptive immune response). All three cases have the potential to contaminate eggs internally by shell penetration, ascending infection, or transovarian contamination. Re-infection of the first and the last case might result in hens that could produce a high frequency of eggs because an immune response with memory was never established (as if never infected). In addition, alternation in surface structures leading to immune evasion might be more significant in natural flocks where houses can contain 8,000-10,000 layers[7] and the life of a flock can be up to 2.5 years. Therefore, re-infection of naturally exposed hens could increase their frequency of SE-positive egg production compared with experimentally inoculated hens.

Effect of Inoculum Size on Egg Contamination

For a hen to become infected with SE, it must initially be exposed to a threshold level of SE. This initial level, in part, could dictate ability of SE to colonize the hen and contaminate eggs. Experimentally inoculated hen studies typically inoculate hens with high level of SE to infection of all hens. This allows clear interpretation of results, but could artificially overestimate the percentage of SE-positive eggs produced by naturally infected hens. This section will discuss this possibility and its implication on the risk assessment.

Hen dose-response to SE

Gast and colleagues often used high doses (9 $\log_{10}$ cfu) of SE to infect hens, which in turn often yields a greater number of contaminated eggs than naturally infected flock studies.[29,46] This

suggests high doses of SE administered to hens experimentally might artificially yield a high frequency of SE-positive eggs compared with naturally infected birds. This is supported by a study conducted by Gast[23] in which SPF hens were inoculated with either 4 or 6 $\log_{10}$ cfu of SE PT14b: post-2 weeks, lower dosed hens produced 2/40 SE-positive pooled egg content samples compared with higher dosed hens that produced 18/39 pooled egg content samples. Therefore, under these conditions, a 2 $\log_{10}$ increase from 4 $\log_{10}$ cfu/hen will be expected to increase the percentage of SE-positive eggs produced by experimentally inoculated hens.

To predict the effect of a further increase, additional studies conducted by Gast were be evaluated. When 9 $\log_{10}$ cfu/hen of SE were used, Gast and colleagues observed similar, if not lower egg contamination frequencies[21;40] compared with 6 $\log_{10}$ cfu/hen.[23] These data suggest a leveling off of the dose-response effect; therefore, infection of hens with 6 $\log_{10}$ SE might yield similar infection and egg contamination potential as 9 $\log_{10}$ cfu of SE or greater. This effect could be due to SE strain differences, as SE PT14b was used for the 6 $\log_{10}$ dosing compared with SE PT13a for the higher dosing.

In the commercial setting, it is conceivable that commercial hens can be exposed to high doses of SE. Henzler and Opitz[53] found that feces from 1 naturally SE-infected mouse contained 5.4 $\log_{10}$ cfu of SE per pellet. These authors also correlated the presence of SE-infected mice and rats with SE-infected flocks. The data suggest that naturally infected flocks could be exposed to similar SE doses as experimentally inoculated flocks and produce similar egg contamination frequencies.

As suggested above, the hen dose-response to SE is unclear. Data from Gast[23] suggest positive correlation between inoculum size and frequency of SE-positive eggs up to 6 $\log_{10}$ cfu/hen. To the contrary, Humphrey et al.[46] observed oral infection of SPF hens inoculated with 3, 6, or 8 $\log_{10}$ cfu of SE PT4 produced 2/57, 0/163 and 0/75 SE-positive eggs, respectively. This suggests, albeit weakly, low doses of SE might be more likely to produce contaminated eggs or that dose does not necessarily correlate with frequency of SE-positive egg production. As expected, 3 $\log_{10}$ cfu elicited an antibody response that was barely above background over 70 days. These hens were clinically normal throughout the trial; however, 1 hen was positive for SE in the liver. When hens were dosed with 6 or 8 $\log_{10}$ cfu, a strong antibody response and clinical symptoms were observed, yet no visceral organs were SE-positive. Therefore, SE levels below the detection of the immune response might be better able to persist in infected tissues compared with a large inoculum that immediately stimulates a strong immune response that could more rapidly clear the SE infection.

Effect of SE dose on SE level within SE-positive eggs

Inoculum size might also affect the numbers of SE deposited within an egg. This is important as a threshold level of SE is probably needed for their growth in eggs.[54] Gast and Beard[40] inoculated SPF hens with 9 $\log_{10}$ cfu of SE6 and found freshly laid eggs harbored 220 SE cells on average. This number is greater than that observed for naturally infected hens, <10 or <20 SE/egg.[28;47] Therefore, experimentally infected hens might produce SE-contaminated eggs that are easier to detect, suggesting the greater SE-positive egg frequency observed for experimentally infected hens is not due only to an actual incidence increase, but also to a lower false-negative rate.

Naturally infected hen studies and false-negative rates

Naturally infected hen studies suggest the frequency of SE-positive eggs is lower than that predicted by experimentally inoculated hen studies. However, the naturally infected hen studies may not detect SE-positive eggs, thereby lowering their observed frequency. This is supported by the findings of Humphrey et al.,[28;46] who determined naturally infected hens produce 1.0 and 0.9% SE-positive eggs typically containing <10 or <20 cells/egg, respectively. To identify SE-positive eggs, the authors of the former article took 10 ml of yolk and 5 ml of albumen and enriched separately, while the authors of the latter study homogenized individual eggs then removed 10 ml for enrichment. With such low numbers of SE within a naturally infected egg, these authors could have missed SE-positive eggs, assuming a typical 50 ml egg. Therefore, the possibility cannot be dismissed that experimentally infected hens may lay SE-positive eggs at similar frequencies as naturally infected hens.

A similar false-negative argument can be used to interpret the results of the Pennsylvania SE Pilot project.[3] This study found approximately 0.02% SE-positive eggs from naturally infected non-molted flocks, suggesting a low frequency of SE-positive eggs produced in the natural egg production setting. The project, begun April 14, 1992, investigated the frequency of SE-positive eggs produced by naturally infected hens. Enumeration methods of SE from eggs are discussed in the textbox. Gast and Holt[55] stated, "Incubating pooled egg samples for 24 h or more provides an opportunity for an initially small SE population to multiply to numbers that are more easily detected using standard enrichment culture methods. After pre-enrichment incubation of egg pools, samples can also be directly plated onto selective agar media to detect SE, but this approach is relatively insensitive for detecting low initial levels of bacterial contamination." Several studies conducted by ARS demonstrate the latter methods used in the SE Pilot Project[3] may have underestimated the prevalence of SE-positive eggs,[23;55-57] particularly if eggs were contaminated with low levels of SE, similar to that found in natural hen surveys in Britain (<10 or <20 cfu/egg).[28;46]

Methods for detection of SE from eggs by PA SE Pilot project[3]

First method: Eggs were collected from flocks, pooled (10/pool), and incubated 48 hrs at 25°C. Ten ml of this mixture was enriched in Hajna tetrathionate (HTT) broth for 24 hrs at 37°C. One ml was then removed and streaked on xylose-lysine deoxycholate (XLD) agar. Second method: In September 1992, the initial incubation was increased from 48 hrs to 72-96 hrs. Third method: In January 1993, the protocol for isolating SE from egg pools was again revised. In the final procedure, 20 eggs were pooled and incubated for 72-96 hrs at 25°C. Following the incubation, the enrichment procedure was replaced with directly applying a streak of the pooled eggs onto XLD and brilliant green agar (BGA) plates and incubated for 24 hrs at 37°C. This methodology was utilized for the remainder of the PA SE Pilot Project.

Concerning the first and second procedure utilized up to January 1993, it is likely these methods would underestimate SE-positive eggs. Gast[23] inoculated pools of 10 eggs with either 5 or 50 cfu SE. These pools were incubated for up to 4 days at 25°C followed by removal of 20 ml into tryptone soy (TS) enrichment broth supplemented with 35mg/L ferrous sulfate (iron) for 24 hrs at 37°C then incubated in tetrathionate brilliant green (TBG) broth (24 hrs at 37°C). When 5 cfu were used, the frequency of isolation from egg pools increased significantly by day 3 of incubation (5/18) and peaked at 4 days (10/18). Therefore, 2 days at 25°C do not appear sufficient for maximal recovery from egg pools under the conditions used in SE Pilot Project.[3]

The SE Pilot Project used HTT broth, a modified form of TBG used by Gast.[23] HTT should better encourage growth of *Salmonella* specifically, yet it is unknown how this would compare to the two enrichment steps supplemented with iron as used by Gast.[23] Pool made little difference when Gast[23] increased it from 10 (11/18 positive) to 30 (10/18 positive) eggs/pool from 5 cfu inoculates. Therefore increasing the pool size from 10 to 20[3] would not be expected to make a significant difference in recovery of SE. In addition, the volume of incubated pooled egg samples transferred to enrichment broth was examined.[23] Transfer of 20 ml yielded 13/18 positive egg pools, yet transfer of 2 ml to either TS or TSB only detected 5/18. Therefore the SE Pilot Project methods utilizing 20 eggs/pool and 10 ml of transferred incubated egg contents would be expected to yield false-negatives. The 2-day incubation at 25^{o}C and the volume of incubated egg pool removed for enrichment suggest the methods employed by Schlosser et al.[3] underestimate the number of SE-positive eggs.

Concerning the third procedure utilized post-January 1993, this methodology for recovery of SE would also likely underestimate the fraction of SE contaminated eggs. Gast[23] inoculated pools of 10 eggs with low levels of SE (>10 cfu/pool) and incubated for 96 hrs at 25^{o}C (preliminary studies by this author found no differences in direct plate recovery (see below) when incubated 3-5 days at 25 or 37^{o}C). A sample was swabbed onto brilliant green agar supplemented with novobiocin (BGAN) and 20 ml was pre-enriched into TSB broth, TT broth and RV broth. Following pre-enrichment in TBS, a sample from the 3 broths was enriched in TT and RV broth. Direct plating (without enrichment, as was done for the SE Pilot Project post-Jan. 1993) identified 47.1% of the positive egg pools, while the three pre-enrichment broths identified 55.9, 61.8, and 64.7% of the positive egg pools, respectively. Enrichment found 70.6 and 79.4% of the positive egg pools from TT and RV broth, respectively.[23] There appears to be an inhibiting effect from mixed eggs cultures.

Gast and Holt[57] found the addition of iron to the mixed (albumen and yolk) egg pools significantly increased SE recovery, suggesting that addition of yolk to albumen does not fully negate the antimicrobial properties of albumen. Also, different SE strains reach varying levels when grown in mixed egg content (up to 1,000 fold differences), suggesting some SE strains are more difficult to isolate from egg pools.[58] The addition of iron to these mixed egg samples negated the observed differences among strains. These data suggest the lab techniques used by the SE Pilot Project[3] underestimate the percentage of SE-positive eggs by 50% or more.

SUMMARY OF EXPERIMENTALLY INOCULATED HEN STUDIES

Overall, the data presented above do not exclude the possibility that naturally infected hens could produce SE-positive eggs at rates similar to experimentally inoculated hens. SE strain, SPF hens, and SE inoculum size could positively bias (overestimate) fractions of SE eggs from experimentally inoculated hens; however, the effect of many of these factors is unknown. Such factors as false-negatives from naturally infected hens, potential of re-infection by naturally infected hens, and ease of SE recovery from experimentally-inoculated hen eggs suggest the naturally and experimentally-infected hens could lay similar numbers of SE-positive eggs. Therefore, we believe experimentally inoculated hen studies are useful in estimating the frequency and SE levels of SE-positive eggs produced by commercial infected flocks. The fact that hens are experimentally infected does not negate the potential use of data from such studies.

However, a legitimate question remains regarding whether such data can represent a probability distribution for the population of commercial hens in the U.S.

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