Hen inoculation dose

Gast and Holt[33] administered 9 $\log_{10}$ cfu/hen, while Shivaprasad et al.[41] 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 the Attachment B1, the difference in inoculum dose would not by itself explain the difference between the *Ey* infection frequencies of the two studies.

Hen age

The PT13a hens inoculated by Gast and Holt[33] were 6-7 months old compared to 9 and 24 month old hens for strains 27A and Y-8P2 inoculated by Shivaprasad et al.[41] It is unclear what the effect of hen age would be on internal egg contamination by transovarian infection routes.[12] The age difference of 6-7 months and 9 months is not likely a factor in the observed differences of *Ey* infected eggs. Within the Shivaprasad et al. study,[41] hen age differences might have an affect on the positional differences observed between strain 27A and Y-8P2 (Table B12).

Hen type

Another notable difference between the two studies was that Gast and Holt[33] used specific pathogen free (SPF) hens and Shivaprasad et al.[41] used commercial hens of the same breed. The literature reviewed above implies SE infection of naïve hens could result in a higher rate of internal egg infection compared with that of commercial hens (see Attachment B1), all other factors being equal. In fact, other factors may not be equal. Differences could include *Salmonella* exposure history, molting status, production husbandry, and the like. While it is possible some of these other factors could cause an increase in internal egg infection for commercial hens over that of SPF hens, it is equally as likely previous *Salmonella* exposure to commercial hens could have decreased the incidence of internal egg contamination in Shivaprasad et al.[41] compared with Gast and Holt,[33] a result not observed (oral infection: 11 vs. 3, respectively). Therefore, 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*) infections. Shivaprasad et al. 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 Shivaprasad et al.[41] We do not know the effect this difference might have on the reported results. This could be a possible explanation for the observed differences.

SE strain

The results from Shivaprasad et al. might suggest that the strain of SE influences the ratio of the numbers of *Ey* or *Ev* to *Ea* infected eggs. These 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 of Shivaprasad et al. cannot be interpreted as *Ev* or *Ey* infections 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* infected eggs versus only 3 *Ey* ones, whereas, for the strain 27A there were 6 *Ea* versus 11 *Ey* infected eggs (Table B12)), could be differences of *Eac* infections 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 in regard to estimating the percentage of eggs that are *Ey or Ev* is discussed below. For the purposes of this risk assessment, data from the Gast and Holt[33] were used to determine the fraction of *Ey* or *Ev* contaminated eggs. Though data of Shivaprasad et al.[41] were considered, they were not explicitly used due to the reasoning above.

*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] This study 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 infection events suggest that these were not penetration events from the OS or contaminating albumen, though these possibilities cannot be negated, 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 when no other egg components are SE contaminated. 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 the contamination of the IS membranes will effect subsequent growth of SE. The IS membranes, composed of an outer and inner membrane, 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. Therefore, the IS membranes are likely to present a physical barrier to SE penetration into albumen and migration to the yolk. *Es* penetration into the albumen, now an *Eaf* infection, 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 *Es* will be less likely to grow compared to *Eaf*, *Eac*, *Ev* and *Ey*. At the same time, *Es* 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 infection

Three additional studies support the notion of *Es* contamination: 1) 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 one 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. 2) Okamura et al.[35] reported hens inoculated with 6.7 $\log_{10}$ CFU IVg produced a total of 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%). 3) Okamura et al.[32] found hens inoculated with 6.7 $\log_{10}$ CFU intravenously (IV) produced a total of 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 infection, with IVg inoculation realizing higher *Es* infection rates compared with oral or IV inoculation.

Es *contamination estimate*

For the purposes of modeling *Es* events**,** it is assumed that the percentage of E*s*-only infected eggs among all infected eggs is equal to 10/(44+10) = 18.5%. It is also assumed that there is no growth within this egg compartment until the event of yolk membrane breakdown YMB. Uncertainty of this percentage is determined assuming the numbers of *Es* only infections and other infections 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

Given the percentage of eggs contaminated via transovarian infection, it is now necessary to separate them into types of infection. This is important as the location of SE within the egg will

determine, in part, the potential and extent of subsequent growth. Five types, based on the location of the initial infection, are identified in this risk assessment: In the yolk (*Ey*); on the vitelline membrane (*Ev*); in the albumen (*Ea*); near the yolk but in the albumen (*Eac*); further away from the yolk but in the albumen (*Eaf*); and in the inner shell membranes (*Es*) (see above)

The percentage of *Es* only eggs is given in the above analysis. The percentage of *Ey* and *Ev* eggs is calculated using data that estimates the percentage of *Ey* infection and total SE-positive infections. These data account for false negative rates as discussed below. The percentage of *Ea* is determined to be the residual incidents. From that residual, the percentage of *Eac* and *Eaf* is assumed as a state of knowledge variable. The method by which the infected eggs were attributed to the different infection sites is described in this section (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, the Gast and Beard[39] and Gast and Holt[33,45] data are used to generate subjective probability estimates of these distributions.[a]

To combine data of these three studies to calculate the fraction of *Ey* or *Ev* eggs, an evaluation of the inoculation protocols used by these studies was conducted. It was therefore assumed that the similar protocols used by Gast and Beard[39] and Gast and Holt[33,45] would produce similar percentages of infected eggs.

To estimate the percentage of *Ey* or *Ev* eggs: 1) the percentage of total infected eggs is needed and 2) the percentage of *Ey* or *Ev* infections is needed. With these two numbers, the percentage of *Ey* or *Ev* eggs of all SE-positive eggs can be calculated. 1) To determine the percentage of total eggs infected, it can be assumed that approximately 16% (22/138) of eggs laid were infected.[39] 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. 2) To determine the percentage of *Ey* or *Ev* egg, it can be assumed that approximately 2.4% (21/874) were *Ey* or *Ev* infected.[45] To account for a false negative, it can be assumed that 6.37% of the eggs were *Ey* or *Ev* infected.

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* infected. 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 infected eggs that are *Ev* or *Ey* infected eggs is assumed to be a state of knowledge variable ranging from 1% to 50%.

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

Of these eggs, a fraction could be *Ey*, where growth is the most rapid. 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 to be 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* infected eggs and n_v is the number of *Ev* infected eggs (assumed not infected in the yolk). *R* is equal to $n_y/(n_y+n_v)$, where

[a] We are cognizant of the possible implications of the Shivaprasad et al.[41] 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 the risk assessment.

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* infections for infected *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).

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

The remaining parameter to be determined is the percentage of *Eac* infections from among *Ea* infections that are not also *Ey* or *Ev* or *Es* infections. An *Eac* infection can be caused by migration of an *Eaf* infection within 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 infected, is limited. The reason for this limitation is as the yolk travels down the magnum, albumen is spooled over the vitelline membrane. As the albumen that could harbor *Eac* infection will be a smaller proportion of the total albumen, *Eac* infections will constitute a lower fraction of *Ea* infections, given that the yolk and vitelline membrane are not infected. As the transit time for the yolk in the magnum is approximately 3 hours, the majority of the transit time in the oviduct will likely result in *Eaf* infections and not *Eac* infections. *Eaf* infection can occur prior to the complete deposition of the inner shell membranes from the isthmus, as the egg transit time will be approximately 1 hour in this section of the oviduct.

Eaf infections would be expected to constitute a greater proportion of the total *Ea* infections unless the magnum is preferentially infected by SE, which could occur for particular SE strains (see SE colonization of the oviduct). As a lower bound, this analysis is assuming as little as 20% of the *Ea* infections are *Eac* based on the belief that *Eac* compartment volume at least constitutes this percentage of the total egg albumen volume. Therefore, the percentage of *Eac* infections from among *Ea* infections is assumed to be a state of knowledge variable ranging from 20% to 50%.

TABLE B13 PERCENTAGES OF CONTAMINATION SITES.

Infection 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

The primary route of internal egg infection by SE is transovarian infection. Eggs can also be infected via "through shell" penetration of the egg surface by contaminating SE. Although this is

not believed to be a common occurrence, the large number of eggs produced necessitates an estimate of the size of this particular hazard. Also, other *Salmonella* spp. beside SE can penetrate the unbroken surface of an egg thereby posing a risk to the consumer. This section begins with a review of the supporting literature followed by data analysis to estimate the percentage of shell penetration events (*Ep*) by SE and other *Salmonella* spp. Spent hen surveys are used to estimate the percentage of *Salmonella* spp. positive flocks and within-flock prevalence. Following these estimates, results from controlled experiments are used to estimate the percentages of surface SE positive eggs and shell penetration events. This process is discussed below.

Mechanisms of shell contamination and egg shell penetration

The process responsible for egg shell contamination by infected birds is not yet 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, i.e. intestinal infection. The egg surface can also be contaminated within the hen reproductive system after formation of the shell, i.e. oviduct contamination. Both methods will lead to contamination of the egg surface and potentially the 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 in important for egg shell contamination. Alternatively, Humphrey et al.[12] found shell-positive eggs could be produced by hens that were fecally negative for SE.

Limitations of data from Schoeni et al.[6]

The data presented by Schoeni et al.[6] suggest SE, as well as other *Salmonella* serotypes, can penetrate the egg shell and deposit high levels of bacteria internally. However limitations of the data must be considered to properly interpret the results of this study.

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, defining a dynamic microbial ecology at the eggshell's surface. The presence of these indigenous fecal bacteria competing for nutrients and living space 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°C, inoculated with *Salmonella* and then placed at 4°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 (see above), this study may overestimate *Ep* (if shell contaminated eggs on a farm are allowed to cool below 35°C before placement at 4°C) or underestimate *Ep* (if shell contaminated eggs on a farm are placed at 4°C before they reach 35°C).

Nevertheless, these data do suggest SE can penetrate the egg shell and deposit viable counts within the albumen and would therefore represent events that could occur. Consequently, the *Ep* results from Schoeni et al.[6] will be used in this risk assessment.

Once *Salmonella* is deposited on the surface of an egg, it must overcome several barriers until it can gain access to the albumen. The shell of the egg is covered by a thin glycoprotein layer that is known as the cuticle. This structure serves to make the shell resistant to water. It also plugs the 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. The bacteria can then cross through the many pores of the outer shell. This action is facilitated by a decrease in external temperature compared with the internal egg

temperature. As the 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. *Salmonella* would likely then be at the surface of the inner shell membranes. To reach the albumen, bacteria would then need to cross the inner shell membranes as discussed above in section: *Salmonella* inner shell membrane contamination.

Frequency of shell contamination

A review of the published literature from experimentally and naturally infected hen data suggests shell eggs can be topically infected from 1 to 53% of 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 one 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, 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 and not that by other *Salmonella* spp. Therefore, the data of Humphrey et al.[46] 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 where possible casual factors include hen breed, SE strain, the immune response, hen age, route of SE contamination, and detection methodology. To estimate the percentage of SE surface positive eggs, the study of Bichler et al.[5] was used (Table B14). This analysis is given in the following section.

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.[41]		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.[46]	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

SE and other *Salmonella* spp. deposited on the egg shell can penetrate this surface and internally contaminate an egg. This assessment used the work of Schoeni et al.[6] to calculate the percentage of SE shell infected eggs that would be penetrated by SE and other *Salmonella* spp.

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 either 4, 25, 35 ºC before inoculation of feces with one of the three *Salmonella* serotypes (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 25 ºC. The study design included a test scenario intended to simulated hatchery conditions (incubated at 35 ºC for 30 minutes, followed by storage at 4 ºC). Eggs were analyzed 1, 3, 7, and 14 post-inoculation. The 7 and 14 day results will not be considered for the purpose of modeling *Ep* because *Salmonella* shell contaminated eggs will typically be removed from the farm environment and washed by 1 week. Therefore, only the egg penetration data collected within the first week is relevant to current egg production practices.

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 in this risk assessment to identify the percentages of through shell penetration events (*Ep*) is given in Table B15. The percentage of *S.* Typhimurium and *S.* Heidelberg penetrating the shell were combined due to the similarity of the data.

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 the results section.[6] At 25 ºC, all *Salmonella* strains grew in feces by 1-2 $\log_{10}$ by day 1 and by 4-5 $\log_{10}$ by day 3 (data were not shown). 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 4 ºC, 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 4 ºC.

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

As with estimating the percentage of SE positive eggs by transovarian infection, no study exists to estimate this percentage directly for shell penetration. This risk assessment used the following approach. To estimate the percentage of SE positive eggs by egg shell penetration this risk assessment first used spent hen data as a proxy to estimate the percentage of *Salmonella* spp.

positive flocks. To estimate the within-flock percentage of *Salmonella* spp. infected hens, data from a spent hen survey was also used. Following these estimates, results from controlled experiments are used to estimate the percentages of surface SE positive eggs and shell penetration events (*Ep*).

The approach of modeling *Ep* infections is similar to the approach that was used for modeling the percentage of SE transovarian infected eggs. However, unlike the latter, we do not have data describing the distribution of the within-flock percentage of hens that are infected with *Salmonella* spp., or even data that can be used to estimate the percentage of flocks that are *Salmonella* spp. infected. With regard to the latter, the only information available is from spent hen surveys which 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
Total	95.4

Some of these differences might be explained by regional and seasonal effects as well as other environmental factors and methodologies used. From these data, it seems reasonable that greater than 90% of the 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. It seems that a large percentage of the flocks could be infected, so that this risk assessment will assume 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 papers quoted above. Waltman et al.[18] report using pooled samples of 3 or 5 ceca, 76% of the flocks had isolations rates of 50% or greater and 37% of the flocks had isolations rates of 75% or greater. The samples were taken from the Southern region of the United States, 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 3700 samples) and the percentages did not differ greatly by state (the largest percentage was 83.3% from a state with 120 samples). By using Equation B1 with an assumed false negative test rate of 10% and 4 ceca per sample (an assumed average value), the percentage of hens infected hens was determined (Table B17).

TABLE B17. ESTIMATES OF THE PERCENTAGE OF *SALMONELLA* SPP. POSITIVE HENS.

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 β. Estimates of values of α and β 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, " and β. The estimated values of " and β are those that minimized the sum of squares of the three differences: $I(0.184|"$, $\beta)$ - 0.24; $I(0.361|"$, $\beta)$ - 0.63; and mean of the beta, "/("+β) – 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 and 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 hens 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($p|\ ''$, β), then $''$ = 0.7230 and β = 7.454, are the values of $''$ 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. for the risk assessment. 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 the 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 " and β was obtained by bootstrapping. A total of 12,000 simulations were generated, where for each simulations, 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 " 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 " and β very large, greater than 20, or very small, close to 0, and thus were excluded. The square root of the 11760 generated values of " 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 are: 1.50942 and 2.23851, which, when squared, equals 27836, 5.01092, respectively, corresponding to and $\mathcal{S}$. The correlation of the square roots of α and $\mathcal{S}$ 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 is the percentage of SE strains from among all *Salmonella* strains infecting hens within a flock that is 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, it is assumed that 2% of the *Salmonella* strains that have infected a flock are SE for this risk assessment. A summary of the assumptions used for modeling *Ep* events is presented below in the Assumptions section.

Biological Reasons Why Contamination Rates Vary Between Flocks, Hens, and Eggs

The estimates and assumptions used in this risk assessment are based on interpretations of the available data. An analysis of this data often revealed that it was equivocal in nature. It was therefore recognized that a detailed understanding of the mechanisms behind disease causation would be important in evaluating equivocal data. This analysis not only confirmed that different interpretations of the data exist among the scientific community, but that due to the diverse genetic nature of SE, variation among studies would be expected. This section provides an analysis of some of the pathogenic mechanism used by SE to contaminate eggs. Diversity among these mechanisms could lead to the diversity seen in the published literature regarding the frequency, level and location of SE within the hen and the egg. These data are not directly used

in modeling; however, they provide a better understanding of why choices were made for the above estimates and assumptions.

SE is a highly adapted pathogen that can survive for extended periods of time within different host backgrounds and the environment. This virulence pattern has been linked to the pathogen's ability to colonize different host tissues, resulting in various potential sites of infection within a single host. To date, there are over 2,000 serotypes of *Salmonella* as determined by carbohydrate-containing lipopolysaccharide (LPS) surface structure and flagella proteins. These serotypes represent an enormous amount of genetic and phenotypic diversity that can cause a range of disease in a variety of host backgrounds. Currently, it is understood that much of the diversity among *Salmonella* clinical isolates is due to: 1) diversity among genes encoding surface structures and 2) diversity among specific virulence mechanisms that alter host cell physiology and intracellular survival.

This section focuses on the role of surface structures in SE colonization of hens and infection of eggs. Flagella, fimbriae, and LPS are three major outer membrane-associated surface structures that are important for the virulence and success of *Salmonella*. These structures are quite genetically diverse, probably due to co-evolution with the host-adaptive immune response. This selective pressure is believed to be the driving force behind the observed biological and genetic diversity seen among bacterial surface structures. Therefore, investigators researching SE have devoted much energy to studying flagella and fimbriae, and to a lesser extent LPS.

In regard to SE contamination of shell eggs, there is much variation in the published literature suggesting a genetically diverse group of SE are capable of being deposited into eggs. Phage type (PT) analysis has identified several strains of SE that can contaminate eggs as well as cause disease in humans. Inter- and intra- PT difference have been associated with different frequencies of egg contamination, suggesting strain differences among SE can effect a hen's ability to produce SE-infected eggs. We believe surface structure variation of flagella and fimbriae, and potentially other surface components such as LPS, could alter SE's ability to differentially colonize various hen organs and egg compartments. This could explain egg contamination level differences and site of egg contamination variation. Below, flagella and fimbriae are discussed in terms of SE contamination of eggs, followed by a discussion of the contribution of SE and hen genotype to variation observed among different studies.

Contribution of SE surface structures in hen colonization

SE colonization of host tissues is a primary step in the infection process of a hen. Successful colonization of intestinal tract and reproductive system can lead to external and internal egg contamination by SE. SE surface structures are important in SE colonization. Therefore, it is hypothesized that genetic diversity among these structures can result in varying levels and location of colonization by genetically distinct SE. Variation among colonization levels and location within the hen could affect the frequency, level and location of egg contamination. Therefore, genetic difference in these surface structures among different SE strains would be expected to produce different results among studies using similar techniques. Below, this risk assessment discusses the role of flagella and fimbriae surface structures in contamination of eggs and variation among studies.

SE flagella

The role of the SE flagella in hens has not been well investigated and published results appear contradictory. Disruption of *fliC* in SE showed a role for flagella in adherence to a human cell line.[48] However, another study demonstrated adherence was unaffected by the absence of this protein.[49] Similar techniques, but different SE strains were utilized, suggesting strain variation could account for the discrepancies between these two studies. However, lab-lab variation could also play a role. Allen-Vercoe et al.[50] showed SE flagella negative mutants were statistically reduced in their ability to adhere to and invade 1 day-old primary chick gut cells in vitro, suggesting a role for flagella in adherence to chick intestinal epithelial cells. In vivo analysis within the 1 day-old chick model showed flagella mutant levels recovered from spleen and liver samples were reduced compared with wild-type ($P < 0.008$), suggesting the flagella could be important in colonization of extra-intestinal tissues such as the reproductive tract.[51]

The role of flagella in colonization of the hen reproductive tract is unclear. However, the heterologous nature of flagella among the *Salmonella* population, combined with differential phase expression, suggest flagella are important in colonization of host tissues. This structure could mediate binding to the ovary or oviduct, as colonization of these tissues can be frequent. Therefore, differences in flagella among strains could in part account for variation seen in the literature regarding frequency and level of egg contamination.

SE fimbriae

The ability of *Salmonella* to utilize different host tissues, resulting in various sites of infection within a single host, has been attributed to the diverse clinical outcome of disease mediated by different *Salmonella* spp. This differential colonization of host tissues is mediated in part by fimbriae, small hair-like appendages located on the bacterial surface. Therefore, fimbriae might be involved in colonization of SE to either the ovary or the oviduct or both. Fimbriae are characterized by their ability to bind host cell-surface compounds and mediate adhesion. This is important as adhesion is a critical step in the virulence of *Salmonella* spp. as it localizes colonization of specific host tissues. Colonization of the hen reproductive system is likely the first step in transovarian infection of eggs. Therefore, colonization of different sections of the hen reproductive tract could explain variation in the level and incidence of SE egg contamination, as well as positional differences in SE egg deposition.

The role of fimbriae in SE colonization of hen tissues is unclear as conflicting studies exist in the literature. Below, we have consolidated published data on SE fimbriae. In vitro evidence for the importance of fimbriae in colonization of the hen reproductive tract is followed by in vivo evidence. This is followed by the role of surface structures in increased yolk membrane breakdown of contaminated eggs. This section attempts to evaluate the role of fimbriae in regard to hen colonization and egg contamination and demonstrate how fimbriae could explain variation among egg contamination studies.

In vitro study of SE fimbriae

To study SE hen colonization and its role in egg contamination, researchers have begun to investigate the relationship between fimbriae and the hen reproductive tissue in vitro. SE was observed to adhere to chicken ovarian granulosa primary cells.[26] These cells were derived from ovarian tissue of healthy adult laying hens thereby representing a more in vivo simulation of ovarian tissue. These authors analyzed microscopically the spatial patterns of binding of 4 SE

PTs to chicken epithelial cells and identified 3 different binding patterns, including an aggregative binding pattern. SE are known to express a fimbrial structure involved in aggregation; however, the molecular determinate for this binding phenotype was not identified in this study. It was found that 11% of PT 8 and 17% of PT 28 strains adhered to mannose-containing epithelial cell surface compounds. The observation that some fimbriae adherence is mannose dependent[52] suggests adhesion to ovarian granulosa cells involve fimbriae for some SE strains.

In a second study, Thiagarajan et al.[53] demonstrated SE adherence and invasion of granulosa cells using the same in vitro tissue culture model. Invasion of this cell type is significant as this behavior could lead to chronic SE colonization and immune evasion in vivo. Furthermore, addition of one purified fimbriae type (SEF14) in a competitive adherence assay resulted in a concentration dependent loss of SE adhesion to granulose cells, suggesting that this fimbrial antigen is involved in binding of SE to ovarian tissue. The involvement of other fimbrial antigens was not characterized and 20 mg/μL of SEF14 protein inhibited 32% adhesion relative to controls, suggesting that other factors are involved in adhesion of ovarian tissue. This observation is the first direct evidence of the role of fimbriae in colonization of the hen reproductive tract.

Researchers investigated the role of SE fimbriae in regard to adherence of gastrointestinal cells. An in vitro adherence assay demonstrated that SEF17 and 21 (two distinct fimbrial types) were important in adherence to INT-407 and Caco-2 human intestinal epithelial cell lines, but SEF14 was not important.[48] Aslanzadeh et al.[54] observed a similar result for SEF21 adherence to mouse intestinal epithelial cells and Thorns et al.[55] found SEF14 to be unimportant in adherence to HEp-2 human epithelial cells. Interestingly, Allen-Vercoe et al.[50] demonstrated SE fimbriae may cooperate to mediate attachment to hen duodenal primary cells. Individual mutations in all five fimbrial operons did not result in mutants showing significant adherence decreases at 1 and 3 hrs. However, when all five fimbrial operons were mutated, this mutant was not significant at 1 hr ($P = 0.791$) followed by closely significant result at 3 hrs ($P = 0.082$). The authors suggest this statistically insignificant result could be due to the lack of fimbrial expression in the control strain, as SE was grown in LB medium post-inoculation, a medium known to poorly induce expression all fimbrial types. This theory is supported by the kinetic changes observed between the 1 and 3 hrs assay, as contact with epithelial cells might induce fimbriae expression by the 3 hr time point. Moreover, the authors note that chicken cells used in this experiment were from 1 day-old chicks, a model that might not properly express hen fimbrial adhesins. Lastly, in vitro analysis found SEF14 mutants to be more susceptible to ingestion to human neutrophils, but not macrophages, compared with wild-type.[56]

These data suggest different SE fimbriae have different roles in pathogenesis, such as adherence to host tissues, and could therefore be important in hen colonization of the gastrointestinal tract. This is significant as gut colonization is often the first step to systemic infection. Therefore, SE better adapted to gut colonization could gain systemic access more quickly and/or more frequently. This could eventually result in infection of reproductive tissue and internal contamination of eggs.

In vivo study of SE fimbriae

To begin to investigate the role of SE fimbrial structures in vivo, researchers used 1 day-old chick virulence and adult hen colonization models. Thorns et al.[56] found no significant

differences at 3 and 7 days between wild-type SE and a SEF14 fimbriae mutated strain in the 1 day-old chick virulence model for their ability to colonize the cecum or persist within the liver or spleen. These data were confirmed by Allen-Vercoe et al.,[51] who also utilized the chick model. They found no differences between wild-type SE and the same strain mutated in all 5 known fimbrial operons for their ability to invade the spleen and liver. However, unlike the former study, they observed a significant difference ($P = 0.001$) between the mutant and the wild-type to colonize the ceca 1 day post-inoculation (a time point not taken in the other study), but not at 2 or 6 days. The chick virulence model does not physiologically represent adult laying hens as suggested above. Chicks will become ill and die at moderate SE oral inoculums (5 $\log_{10}$) compared to experimentally inoculated adult hens (>18 weeks old) that typically show no clinical signs of illness with 9 $\log_{10}$ SE. Furthermore, chicks do not have developed immune systems, further emphasizing the age difference. It is therefore difficult to interpret these results as these data only are informative regarding the role of fimbriae in young chicks.

Thorns et al.[56] found 20-week old hens inoculated with 8.7 $\log_{10}$ cfu SE or SEF14- mutant showed no significant differences between the level of colonization of the liver, spleen and ovary. However, 1 week post-inoculation, SEF14 mutants were fecally shed more frequently (25/67) than birds inoculated with SE wild-type (12/67). These data suggest SEF14 is important for infection of the gastrointestinal tract, yet not for colonization of the liver, spleen and ovary. Unfortunately, this group did not look at any other fimbrial mutants and therefore these data do not negate the role of fimbriae in colonization of the ovary or oviduct. The need to investigate multiple SE fimbrial types is important as *S.* Typhimurium fimbriae are thought to act synergistically to mediate adhesion.[57] This notion is also support by Allen-Vercoe et al.,[51] as discussed above.

Thiagarajan et al.[27] infected laying hens with either a SE strain expressing fimbriae SEF14 and 21 or an SE strain lacking these proteins. The former strain was found to colonize 28.6% (10/35) of hen reproductive organs and the latter strain was found to colonize 17.1% (6/35). These differences were not statistically significant; however, they allow the possibility that fimbriae are involved in colonization of the hen reproductive tract. Additionally, there were methodological problems complicating the interpretation of these results.

First, experimentation conducted to identify the effect of functional loss of a particular protein must assume all other characteristics between the two strains are equal to effectively evaluate the differences of interest (in this case the presence or lack of fimbriae SEF14 and 21). This is achieved by making a controlled and defined mutation within the gene(s) encoding the protein(s) and then genetically characterizing the mutant to confirm the desired mutation. These two isogenic strains (the wild-type and the mutant created from the wild-type strain) allow clear interpretation of the results. Thiagarajan et al.[27] did not use isogenic strains, but rather characterized two environmental SE PT 8 strains for the presence or lack of SEF14 and 21. Therefore, innate differences between the two strains could have affected the observed results.

Second, SE can differentially express fimbriae. Because defined mutations were not made in the fimbriae-minus strain, the fimbrial expression state of this strain was unknown during this experiment.

Third, the authors did not investigate the levels of colonization (only the presence) of SE within the reproductive tract. Therefore, levels of SE within the reproductive tract could have been reduced by the absence of SEF14 and 21. Also, as the oviduct and ovary data were combined, it is unknown if loss of these fimbriae altered colonization of these specific tissues.

Fourth, all fimbria types need to be expressed for full virulence of *S*. Typhimurium. Deletion of one particular fimbrial gene resulted in a 3-fold murine model LD_{50} increase. However, deletion of 4 fimbria types led to a 26-fold increase. This suggests defined mutations in all SE fimbrial genes might be needed to observe a demonstrable result within the hen colonization model.[57] This could explain the small difference observed between the two strains (28.6 and 17.1%). Due to these issues, the role of SE fimbriae in adult hens still remains unclear.

In addition, Rajashekara et al.[58] investigated the ability of fimbriae mutants to colonize the liver of chickens. Interestingly, one of their mutants was unable to colonize the liver at wild-type levels suggesting a role for fimbriae in colonization of extra-intestinal tissues. This mutant was deficient in the same fimbrial antigen (SEF14) as a mutant used by Thiagarajan et al.[27] above. Yet when they investigated liver colonization they found a small, but statistically significant difference. These data suggest SE strain differences could explain some of the variation of results from different studies.

The above in vitro and in vivo data demonstrate an inconclusive role for fimbriae in colonization of the hen reproductive tract. However, SE have been observed to colonize hen ovarian tissue, oviduct tissue and vaginal tissue more frequently than five other food-borne related *Salmonella* serotypes and ultimately produce more contaminated eggs.[32,34,35] This increase could be due to specialized fimbriae produced by SE during infection. In support of this, minor mutations within the structural components of fimbriae have been known to result in increased adhesion and colonization of different host tissues by *E. coli*.[59,60] In this example, commensal *E. coli* previously restricted to a mammalian intestinal niche, acquired the ability to bind urinary tract cells, thereby altering its normal colonization site and ability to cause disease. This alteration in binding specificity is due to two amino acid mutations thought to occur spontaneously. A similar scenario could have occurred with SE attachment to hen reproductive tissue.

Role of fimbriae and flagella in yolk contamination

The data present an inconclusive role of fimbriae and flagella in colonization of the hen reproductive system. However, these structures do appear to play a role once they become internal residents of an egg. These structures appear to allow SE to gain access to the nutrients of the yolk more quickly than those bacteria lacking these structures. SE that have access to the internal yolk contents could grow exponentially given reasonable environmental conditions. This would pose a substantially greater risk to consumers than SE-infected eggs that could not grow are increased rates.

The fimbrial structure SEF17 increases the likelihood that SE can invade the yolk and motility (imparted by the flagella) is important for rapid invasion of the yolk.[61] Cogan wrote, "Non-motile serovars and [defined motility] mutants of *Salmonella* were introduced into the albumen of eggs. No multiplication took place until after 21 d storage at 20°C, by which time the vitelline membrane was sufficiently porous to have allowed iron and other nutrients to have diffused from the yolk into the albumen. Motile strains were able to enter the yolk and multiply within 4 days. SEF17 appear to be implicated in bacterial attachment to the vitelline membrane. These [flagella and fimbriae] are not an absolute requirement for yolk invasion, but strains able to express them are more likely to enter the yolk." These data suggest SE unable to express these

structures would demonstrate varied abilities to enter the yolk. Therefore variation within the SE population to enter the yolk at a given moment would be expected.

The data above, though admittedly somewhat unclear, suggest that it is possible flagella and fimbriae play a role in SE colonization of the hen reproductive system and contamination of eggs.[53] These genetically diverse and differentially expressed structures therefore may explain the variation observed in the literature among studies investigating the frequency, level and location of SE within an egg. To this end, several studies have observed different sites of egg contamination, most notably contamination of the yolk vs. the albumen (Table B12). It is currently hypothesized these differences in contamination sites within the egg are conditional upon SE colonization of the ovaries or the oviduct, respectively. Structural fimbrial or flagella differences or expression could account for the variations observed by investigators using different SE strains. Some strains might be better adapted for ovarian colonization, explaining why some investigators observe more yolk contamination as compared to albumen. Conversely, some SE strains might adhere better to oviduct tissue. There is evidence in the literature that different strains of SE adhere differentially to the hen reproductive tissue[25,26] and strain differences accounting for varying incidence of egg contamination.[39,41] These data taken together suggest that fimbrial or flagella strain differences could explain frequency of egg contamination differences as well as site of egg contamination variation.

Contribution of hen and SE genotypic variation

Epidemiological evidence suggests certain PTs of SE are more frequently associated with egg contamination than other SE strains. This suggests there is a genetic component that allows these strains of SE to better colonize and/or contaminated eggs. As evidenced above, genetic diversity among SE surface structures could result in increased egg contamination. Below, data is presented that supports variation in hen and SE strain genotype can alter the frequency of SE-positive egg production. In turn, this suggests genotype could also alter the level and the location of SE with a contaminated egg.

Hen breed

To investigate the role of hen genotype in regard to egg contamination, Lindell et al.[62] inoculated 4 breeds of hens with the same SE strain to investigate genotypic breed differences in response to SE infection. Significant differences in the number of SE-positive eggs laid within the first 14 days were realized depending on the infected breed (45-week old hens). Interestingly, there were no differences in SE ovarian colonization between the two breeds; however, they did not culture oviduct tissue. Protais et al.[22] found a similar egg contamination breed dependency. Breed L2 (20-week old) produced 15% (48/317) SE-positive eggs compared with 4 other hen lines (1/330, 0/210, and 0/181). This susceptible breed had the greatest SE positive percentage of intestinal and extra-intestinal cultures, including 9/16 ovary and 8/16 oviduct SE-positive cultures. These data suggest various hen breeds are innately more or less susceptible to SE infection and egg contamination.

Though the mechanism(s) is not known, studies suggest the immune response is involved in these observed differences.[22,63,64] Therefore, hen breeds that can mount an effective immune response would likely clear the SE infection more quickly and produce less SE contaminated eggs. However, the predictive relevance of hen breed for various disease factors is somewhat

unclear. Three studies, utilizing the same 4 hen lines and the same SE PT4 strain, recorded differences in susceptibility or resistance of these hens.[22,65,66] For instance, Duchet-Suchaux et al.[66] concluded hen line Y11 was most resistance to SE cecal colonization among the four hen types, yet Girard-Santosuosso et al.[65] showed Y11 was one of the lines more susceptible to higher SE levels in the ceca and liver. Some of these differences are dependent on the criteria used to determine susceptibility or resistance; however, others cannot be explained so easily. Nevertheless, all three articles, under different criteria, predicted hen line PA12 as an SE resistant line, suggesting that genotypic differences between hen breeds can mediate various disease factors. Therefore, hen breed might affect the magnitude and incidence of the immune response, thereby modifying the frequency of SE contaminated eggs among different flocks.

SE strain differences

Another factor that could affect the frequency of SE positive eggs is the infecting SE strain. Hinton et al.[67] and Barrow et al.[68] demonstrated increased mortality of day old chickens with SE PT4 compared with SE PT 6, 7, 8, 13a. Additionally, these differences extend beyond PT characterization. Shivaprasad et al.[41] demonstrated variations in egg contamination within SE PT8 strains experimentally inoculated into the same hen breed. Oral inoculation of 4 $\log_{10}$ cfu of SE produced contaminated eggs at frequencies of 2.7% (6/221) from albumen and 0.0% (0/221) from yolk. However, a similar experiment (6 $\log_{10}$ cfu oral infection) with a second PT 8 strain resulted in SE contaminated eggs at frequencies of 1.9% (6/314) from albumen and 3.5% (11/314) from yolk. Also, a third SE PT 8 strain resulted in no SE-positive eggs. Furthermore, Gast and Beard[39] observed both inter- and intra-PT differences when adult hens were orally inoculated with SE. Two trials in which hens were infected with PT 8 strains produced 0.0 or 1.4 % SE-positive eggs, while hens infected with PT 13a produced 0.4 or 8.1% SE-infected eggs. These data are supported by increased severity of infection using a chick virulence model inoculated with SE PT4 over other PTs.[69] Experiments by Lock and Board[70] and Gast and Holt[71] suggest different PTs have different abilities to grow and persist in egg albumen. Therefore some of the difference seen above could be explained not by frequency of egg contamination, but by survival and detection following contamination. These data suggest inter-PT differences as well as intra-PT type differences can result in variation among the frequency of SE-positive eggs produced and even result in variation about the location SE is deposited within the egg.

Hen breed and strain differences can affect the incidence of SE-positive egg production and likely affects the level and location of SE within the hen. This could be due, in part, to the hen's immune system and the effect SE has upon that immune system.

This risk assessment, however, cannot predict what percentage of hen and/or PTs will produce more contaminated eggs in a particular setting. For instance, a more virulent SE PT (defined by invasion into host cells) might be better at initially contaminating eggs; however, might elicit a stronger immune response. This strain could be cleared faster, thereby producing more SE-positive eggs initially, but less over time. Alternatively, a less virulent strain might better colonize a hen by remaining below the threshold of immune detection. This strain would contaminate eggs less frequently; however, persist in the hen for longer periods of time. Therefore breed and strain differences will affect egg contamination frequency; however, the effect can not be predicted from the available data.

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 one 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 the 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 a 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 $\therefore'$ 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-infected eggs, q, that a 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 infections of types *Ey*, *Ev*, *Eac*, *Eaf*, and *Es*, are determined as follows:

    a) The percentage, q_s, of eggs that are *Es* infections (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* infections, and n_s is the number of *Es* infections that are not *Ea*, *Ev* or *Ey* infections 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* infected eggs that are *Ev* or *Ey* infected eggs is assumed to be a state of knowledge variable ranging from 1% to 50%.

    c) The percentage, $q_{y|(v,y)}$ of *Ey* infected eggs from among the *Ey* or *Ev* infected 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

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* infections among *Ea* infections is assumed to be 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 infected 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* infected is modeled in a similar fashion as that for the percentage of eggs that are SE-infected through 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

$$z_{j'} = z_j + \frac{K_4 (z_j^3 - 3z_j)}{24}, \; j = 1,2 \qquad \text{(B7)}$$

c) distributed as a beta distribution, beta($p|\alpha = 2.23315$, $\$ = 4.914942$). Values of α' and $\$'$ reflecting the uncertainty of α and $\$$ 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

where κ_4 is the kurtosis. For $\alpha^{1/2}$, $\kappa_4 = 0.22$ and for $\$^{1/2}$, $\kappa_4 = 0.51$. These adjusted values, $z_{j\mathbb{N}}$ are multiplied by the corresponding standard deviation (0.210 for $\alpha^{1/2}$ and 0.3605 for $\$^{1/2}$), added to the corresponding mean values (2.23315 for $\alpha^{1/2}$ and 4.914942 for $\$^{1/2}$), and then squared to calculate the simulated values of α' and $\$'$.

d) The percentage, q, of shell-infected 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 $(q(1-q)/592)^{0.5}$.

e) The percentage of shell-infected eggs that become *Ep* infected depends upon the strain of *Salmonella*. If the strain is a SE strain, the percentage is 37.5% (6/16); if the strain is not SE, the percentage is 21.9% (7/32). The uncertainty of these percentages is accounting for generating random variables that are normally distributed with mean equal to the percentage, w, and standard deviations equal $(w(1-w)/n))^{0.5}$, 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 well 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 of the results; 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 compared on the basis of the: 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 Strain On Egg Contamination

Many different types of SE exist environmentally. Some of these bacteria might be better adapted to infect hens or contaminate eggs. For experimentally inoculated hen studies,

investigators will typically use a SE strain known to be relevant to the particular research, i.e. a strain associated with human illness or egg contamination. This strain may be used multiple occasions to minimize variability between experiments. As different strains likely have different effects on hens and the contaminated eggs they produce, the results obtained by analysis of a single strain may or may not be representative of naturally infected hens.

Multiple studies have utilized various SE strains to experimentally inoculated hens to determine the frequency of SE-positive eggs produced. This discussion will focus on the seminal work of Gast and colleagues as this risk assessment utilizes much of their work. These authors typically use one SE strain (PT 13a, SE6) and one 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] Based on the small number of strains described in the previous statement, it appears that SE6 is capable of increased egg contamination in this hen breed. However, it is unknown how representative this strain truly 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 be 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. 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 infect eggs[32,35,37] are likely absent from the more common *Salmonella* strains harbored by hens. This is supported research demonstrating that hen immunization with a modified live *S.* Typhimurium did not decrease SE-positive egg contamination when challenged with SE.[72] In fact, SE positive cultures from reproductive tissues, ceca, intestinal tissues as well as other viscera were not statistically different between immunized hen 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 circumstance 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 into their environment exposing 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 strain. However, the ability for experimentally inoculated hens compared to naturally infected hens to mount an effective response against SE will differ between the two populations.

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 (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.[12,33,41,73-75] Though little is known regarding 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 of the vaccine results in a quicker and more sustained antibody response.[72,76] 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 therefore 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, phase variation (altered regulation of surface structures) and even change their phage type (PT) status (suggesting an alteration in LPS). 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 past inoculation under the experimental conditions, re-infection unlikely alters SE-positive egg production in experimentally inoculated hens to a significant extent.

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, those 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), they might become contaminated by outgrowth of SE (developing an adaptive immune response with memory), or hens might become chronically colonized at low levels (no adaptive immune response). All three cases have the potential to internally infect eggs by shell penetration, ascending infection, or transovarian infection. 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, it must initially be exposed to a threshold level of SE. This initial level, in part, could dictate the pathogen's ability to colonize the hen and infect eggs. However, this dose-response is unclear in hens. Experimentally inoculated hen studies typically inoculate hens with high level of SE to infection of all hens. This allows clear interpretation of results. Inoculation of hens with high levels of SE 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 typically used high doses (9 $\log_{10}$ cfu) of SE to infect their hens, which in turn often yields a greater number of contaminated eggs than naturally infected flock studies.[29,46] This suggests that high doses administered to hens experimentally might artificially yield a high frequency of SE-positive egg compared with naturally infected birds. This notion is supported by a study conducted by Gast[24] 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 there conditions, a 2 $\log_{10}$ increase from 4 $\log_{10}$ cfu/hen will 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 can be evaluated. When 9 $\log_{10}$ cfu/hen pf SE were used, Gast and colleagues observed similar, if not lower egg contamination frequencies[21,39] compared with 6 $\log_{10}$ cfu/hen.[24] These data suggest a leveling off of the dose-response effect and therefore infection of hens with 6 $\log_{10}$ SE might yield similar infection and egg contamination potential as inoculation with 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[77] found that feces from one 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. These data suggest naturally infected flocks could

be exposed to similar SE doses as experimentally inoculated flocks and therefore produce similar egg contamination frequencies.

As suggested above, the hen dose-response to SE is unclear. Data from Gast[24] suggest positive correlation between inoculum size and frequency of SE-positive eggs up to 6 $\log_{10}$ cfu/hen; however, this issue remains unclear. 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, one 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 was 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 growth of SE within eggs.[78] Gast and Beard[39] inoculated SPF hens with 9 $\log_{10}$ cfu of SE6 and found freshly laid egg could harbored 220 SE cells/egg on average. This number is greater than observed for naturally infected hens, <10 or <20 SE/egg.[29,46] Therefore, experimentally infected hens might produce SE-infected 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 a lower false negative rate.

Naturally infected hen studies and false negative rates

Naturally infected hen studies suggest that the frequency of SE-positive eggs is lower than that predicted by experimentally inoculated hen studies. However, the naturally infected hen studies likely missed SE-positive eggs, thereby lowering their observed frequency. This notion is supported by the findings of Humphrey et al.,[29,46] 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 another naturally SE-infected survey, the Pennsylvania SE Pilot project.[3] This study found approximately 0.02% SE-positive eggs from naturally infected non-molted flocks, suggested a low frequency of SE-positive eggs produced in the natural egg production setting. The project was begun April 14,

1992 and investigated the frequency of SE-positive eggs produced by naturally infected hens. Enumeration methods of SE from eggs are discussed in Textbox 2. Gast and Holt[79] 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 1995 PA SE Pilot Project[3] would underestimate the prevalence of SE-positive eggs,[24,55,71,79] particularly if eggs were contaminated with low levels of SE, as appears likely based on the British natural hen surveys (<10 or <20 cfu/egg).[29,46]

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 for 48 hrs at 25°C. Ten mL of this mixture was then 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 Sept., 1992 the initial incubation was increased from 48 hrs to 72-96 hrs. Third method: In Jan. 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.

In regard to the first and second procedure utilized up to January 1993, it is likely these methods would underestimate SE-positive eggs. Gast[24] 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). These authors found that when 5 cfu were used, the frequency of isolation from egg pools increased significantly by the 3rd day of incubation (5/18) and peaked at 4 days (10/18). Therefore, 2-days at 25°C are not sufficient for maximal recovery from egg pools under the conditions used in PA SE Pilot Project. Gast[24] did two enrichment steps (compared with the one above) and when 50 cfu were used, they found 17/18 pools SE-positive (94.4%) after 4 days of incubation.

In fact, less than 1/4 of the 5 cfu inoculated pools were detectable by 3 days post-inoculation. Therefore, the size of the initial contamination and length of incubation are critical factors in detecting SE from pooled eggs. However, it is unclear how the enrichment steps might have affected the two protocols. The PA SE Pilot Project used HTT broth, a modified form of TBG used by Gast.[24] HTT should better encourage growth specifically of *Salmonella*, yet it is unknown how this would compare to two enrichment steps supplemented with iron as used by Gast.[24] Pool size hardly made a difference when Gast[24] increased the pool size 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. Also, the volume of incubated pooled eggs sample transferred to enrichment broth was examined.[24] Transfer of 20 mL yielded 13/18 positive egg pools, yet transfer of only 2 mL to either TS or TSB only detected 5/18 each. Therefore the PA SE Pilot Project methods utilizing 20 eggs/pool and 10 mL of transferred incubated egg contents would be expected to yield false negatives. Therefore, the 2-day incubation at 25°C and the volume of incubated egg pool removed for

enrichment suggest the methods employed by Schlosser et al.[3] would underestimate the actual number of SE-positive eggs.

In regard to the third procedure utilized post-January 1993, this methodology for recovery of SE would also likely underestimate the fraction of SE contaminated eggs. Gast[24] inoculated pools of 10 eggs with low levels of SE (>10 cfu/pool) and incubated for 96 hrs at 25°C (preliminary studies by this author found no differences in direct plate recovery (see below) when incubated 3-5 days at 25 or 37°C). A sample was swabbed onto brilliant green agar supplemented with novobiocin (BGAN) and 20 mL was removed and 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 PA 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 pool respectively. Enrichment found 70.6 and 79.4% of the positive egg pools from TT and RV broth respectively.[24] Clearly there is still an inhibiting effect from mixed eggs cultures. Gast and Holt[71] found that 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, particularly the iron-chelating protein ovotransferrin.[55,79,80] Also, different SE strains reach different levels when grown in mixed egg content (up to 1,000 fold differences), suggesting some SE strains are more difficult than others to isolate from egg pools.[81] The addition of iron to these mixed egg samples negated these observed difference among the strains. These data suggest the lab techniques used by the PA SE Pilot Project[3] would underestimate the true 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. The 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 are 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 two study groups 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 their potential information with respect to determining possibilities for a risk assessment. However, a legitimate question remains regarding whether such data can represent a probability distribution for the population of commercial producing hens in the U.S.

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