ANNEX A

General Introduction to the Annexes

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INTRODUCTION

The purpose of this introductory annex is twofold: First, it provides an overview of the content of subsequent annexes (Annexes B through I) that give the rationale for the model used in the risk assessments. Included is a description of data and analysis procedures used for determining the distributions and values of parameters for the risk assessment models. The data analyses in Annexes B through H are inputs to the exposure assessment model for eggs from farm to table. The modeling applications of the results of these data analyses are described in the Exposure Assessment (chapter 3). The model predicts, as final outputs, the frequency and extent of *Salmonella* Enteritidis (SE) or *Salmonella* spp. contamination of servings of eggs or egg products. Annex I describes the data from epidemiologic investigations of foodborne salmonellosis and the procedures used in developing the FAO/WHO dose-response model.

The second purpose of this introductory annex is to provide background information on two subjects, knowledge of which is required to understand better the information presented in subsequent annexes. The first of these subjects is a comprehensive "picture" of the biology relevant in developing a risk assessment model for SE in eggs. The second of these subjects is a description of variability and uncertainty in risk assessment inputs. In the Introduction to these risk assessments we stated that one of our goals was to separate variability and uncertainty. There are many terms researchers use in describing uncertainty. For our purposes, it is sufficient to classify the types of uncertainties into two broad categories: uncertainty calculated from the data; and "state of knowledge" uncertainty in the absence of data.

OVERVIEW OF ANNEXES B THROUGH I

Annex B provides information about the prevalence of SE-contaminated flocks and eggs in the United States. Molting of flocks and penetration of *Salmonella* through the outer shell of the egg are considered as factors contributing to SE prevalence. The factors that affect prevalence of *Salmonella* in eggs also might affect the levels of the initial contamination; however, we are unaware of data to estimate whether such a correlation exists.

Annex C provides information about the initial contamination level of SE in shell eggs, distinguishing levels occurring between yolk and albumen. The amount of growth of SE cells depends upon their growth kinetics, which in turn depends upon the internal temperature of the egg. To model the effects of time and temperature storage scenarios on the levels of SE contamination, it is necessary to model the rate the egg cools and growth kinetics of SE in the egg as a function of temperature. Hence, Annex D describes an exponential cooling rate model that was developed to estimate the internal temperatures of eggs, while Annex E describes the models used to estimate the growth kinetics of SE in shell eggs as temperatures change. These models were used to model growth of *Salmonella* spp. in eggs for various time/temperature storage scenarios.

If contaminated eggs are broken and contents used in producing liquid egg products, then *Salmonella* within the eggs will contaminate liquid product. *Salmonella* spp. on the exterior of the shell during the breaking process may also contaminate liquid product. Annex F presents an estimate of the distribution of *Salmonella* spp. levels in liquid egg products immediately before

pasteurization based on an analysis of data collected from the FSIS Egg Baseline Survey of *Salmonella* spp. in liquid egg product.

The results from Annex F together with predictions based on the models described in Annexes B to E allowed modeling the distributions of *Salmonella* levels in liquid egg products for various time/temperature scenarios. The effect on the distribution of *Salmonella* levels in liquid product if eggs are from SE-free flocks versus those for flocks assumed not to be SE-free can be evaluated for given scenarios of handling eggs before pasteurization. This is important because performance standards, which essentially specify a required probability of assuring no viable *Salmonella* cells after pasteurization for given conditions, are dependent upon the estimated distribution of *Salmonella* levels in pre-pasteurized product.

In addition to the above modeling, we also modeled risk that exists today under present regulatory requirements. Annex G presents data and development of inactivation models for different types of egg products and shelled eggs.

Annex H describes how data from the USDA Continuing Survey of Food Intake by Individuals (CSFII) were used to identify amount and frequency of egg and egg product consumption. These data combined with estimates of the level of *Salmonella* in a serving of eggs or egg products completes the exposure profile.

Annex I presents a report prepared by a Joint Expert Meetings on Microbiological Risk Assessment on the Joint FAO/WHO Risk Assessment of *Salmonella* spp. in Eggs and Broiler Chickens. The dose-response model for non-typhoid salmonellosis presented in the report was used in these risk assessments. The technical details of the methodology cited in the FAO/WHO report are not fully transparent; thus, while the derived dose-response model was used here to compute the probabilities of illness, the procedures used for deriving this model cannot be endorsed by FSIS unless further documentation is provided.

BIOLOGICAL CONCEPTS RELEVANT IN DEVELOPING A RISK ASSESSMENT MODEL FOR SE

Current data demonstrate differences in the incidence of SE egg contamination, SE levels, and growth kinetics by site of contamination within the egg. Development of models for predicting such values were based largely on data from studies with experimentally inoculated hens or eggs. Several biological concepts were significant in development of the data analysis approaches used for the risk assessment of SE in eggs, the most important of which are briefly described below.

Describing contamination of eggs with SE

The growth potential, frequencies of occurrences, and SE levels in eggs depend on the site of contamination during egg formation within the hen (vertical transmission) or after lay (horizontal transmission). Growth potential as supported by the availability of nutrients may be dependent on the site where SE contaminates the egg. We identified six types of SE contamination events (*Ex*, where *x* identifies the site of egg contamination).

1) SE can be vertically transmitted within the hen (Figure A1), migrating to, and colonizing the ovary and oviduct tissues. SE can contaminate the ovule or yolk contents before release from the ovary (*Ey*), as described in Figure A2 and in the text box below.

2) While within the ovary or during release of a yolk from the ovary follicle into the opening of the oviduct (infundibulum), SE can contaminate the vitelline membrane of the yolk (*Ev*).

3) As the yolk descends along the oviduct where the first layers of albumen are laid down around the yolk, SE can contaminate the albumen close to the yolk (*Eac*).

4) As the forming egg further descends along the magnum of the oviduct where the outer layers of albumen are laid down, SE can contaminate the albumen far from the yolk (*Eaf*).

5) As the inner shell membranes are laid down, SE can contaminate the inner shell membranes by vertical transmission (*Es*).

6) SE can contaminate the exterior surface of the shell by horizontal transmission after lay (*Ep*).

Contamination events (*E*) for SE within shell eggs are either vertical or horizontal.

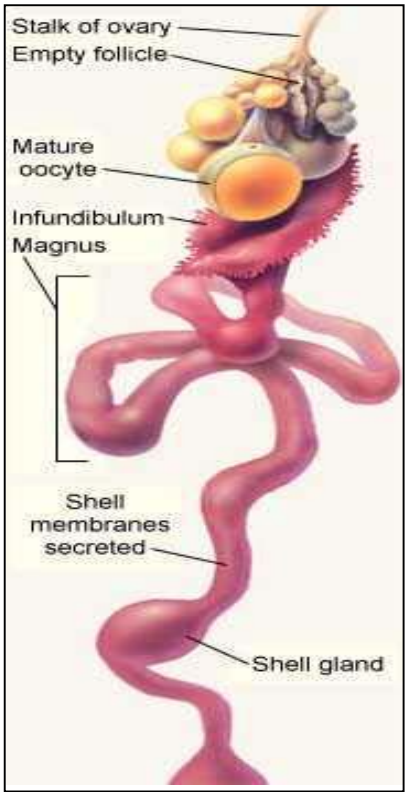

FIGURE A1 ANATOMY OF THE HEN REPRODUCTIVE TRACT (SOURCE:HTTP://CHICKSCOPE.BECKMAN.UIUC.EDU/EXPLORE/EMBRYOLOGY/DAY05/OVARY.HTML)

Type of Event	Contamination Site	Transmission
Ey	In the interior yolk (*y*) contents	Vertical
Ev	On the vitelline membrane surface, (*v*) but not yolk interior	Vertical
Eac	Within the inner layer of albumen close to the yolk	Vertical
Eaf	In the outer albumen far from the yolk	Vertical
Es	In or on the inner shell membranes	Vertical
Ep	Penetrating egg from outside environment	Horizontal

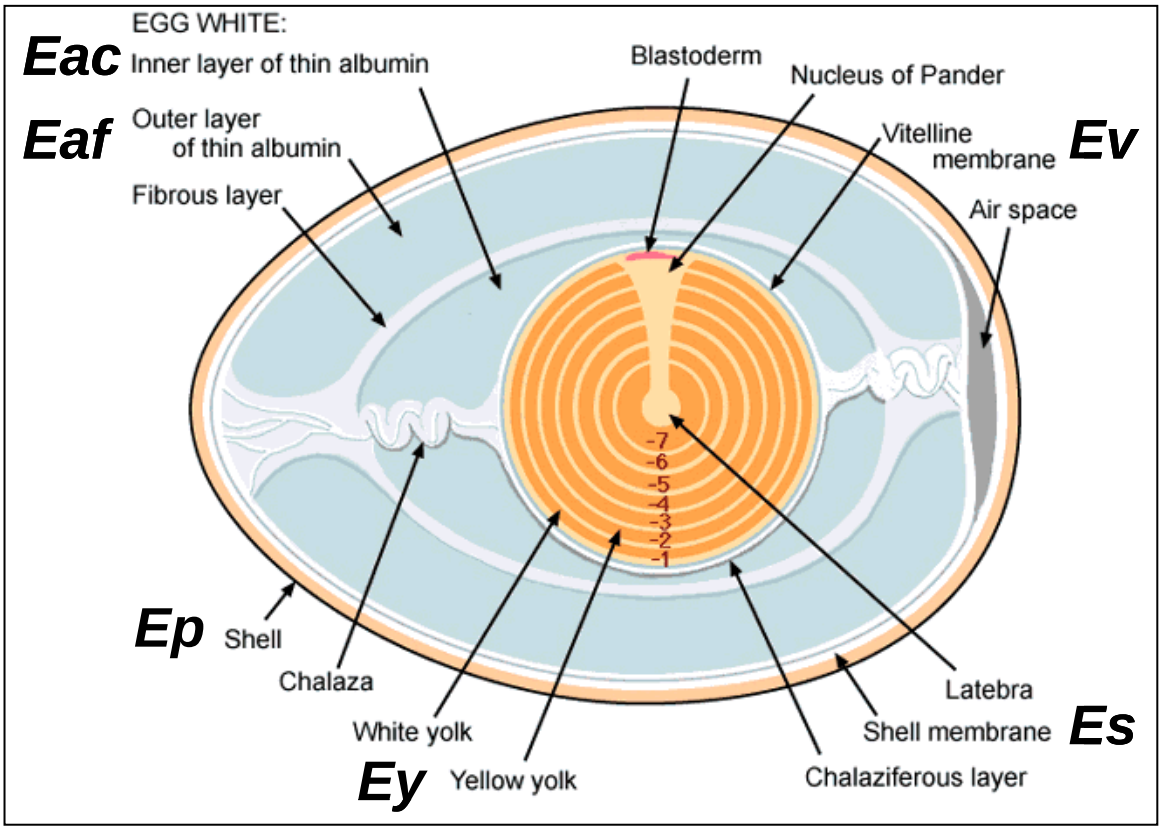

FIGURE A2 ANATOMICAL PICTURE OF EGG COMPARTMENTS THAT CORRESPOND TO POTENTIAL SE CONTAMINATION SITES *EY*, *EV*, *EAC*, *EAF*, *ES*, AND *EP*. (SOURCE:HTTP://CHICKSCOPE.BECKMAN.UIUC.EDU/EXPLORE/EMBRYOLOGY/DAYO1/THE_SHELL.HTML).

Initial growth and physiological state of SE in eggs

Growth in first 24 hours after lay

Humphrey[1] described a 10-fold increase in the number of SE cells within the first 24 hours after lay, explained by hypothesizing that SE is able to utilize internal reserves of iron and grow at neutral pH. We do not assume a particular phase of *Salmonella* growth associated with the first 24 hours after lay. It is possible that growth rates within the first 24 hours are different from those after the first 24 hours; however, no data are available to provide information on the "true" growth curve in albumen. The belief that SE growth is possible in albumen beyond 24 hours played a crucial role in developing the exposure assessment model.

Lag phase - physiology

A difficulty of interpreting data from experimental infection[a] of hens or contamination of eggs is that SE cells prepared for experimental inoculation are often in stationary phase and subject to a lag phase of unknown magnitude. We believe that naturally contaminating SE would behave differently from experimentally inoculated cells. Part of the difficulty of determining growth of *Salmonella* in the egg is the fuzzy picture of the status of the growth phase of the SE within the egg, in particular the physiological state of the SE in the hen before invading the forming egg and immediately after invasion.

Virtually no data exist to characterize lag phase durations or physiological states of SE (or changes of them within the hen or within naturally contaminated eggs) before and after the egg is laid. However, information regarding these features is important, as the lag phase, dependent on the bacterial physiological state and change of environment, will determine the time before SE growth within the egg begins. This information could explain the variability regarding the quantity of SE within young eggs laid by naturally infected hens.[2;3] Explanations of these results depend upon knowledge of the possible growth that could occur before the egg is laid, which in turn depend upon knowledge of the physiological states of SE cells as the cell's environment changes from hen to egg.

The physiological states of SE before deposition into the egg will partly determine length of lag phase as transition into a new environment requires time for SE cells to adjust before growth. We assumed SE cells within the ovary or oviduct are not in the exponential phase of growth:

1) SE-infected hens do not typically demonstrate clinical signs of illness or slowing of egg production rate, suggesting SE growth is controlled by the hen, the bacteria, or a combination thereof.

2) The internal host environment contains limited free iron, likely prohibiting rapid growth of SE colonizing the surface of reproductive tissues.

3) The majority (>90%) of *Salmonella* might be located on the surface of infected tissue as demonstrated by colonization of the mammalian gastrointestinal tract.[4] This suggests most infecting SE could colonize the hen's reproductive tissues as biofilms or microcolonies in which few cells are capable of leaving lag phase before lay.

Taken together, the above points suggest SE would not typically be in exponential phase during colonization of the ovary or oviduct. On the other hand, SE cells are capable of invading into ovarian cells[5] and are likely to do the same in the oviduct. This process results in rapid SE growth within the host cell and release of immune activating and chemotactic chemicals. SE cells emerging from invaded host tissue could be in an exponential phase of growth and, if deposited within an egg, adapt quickly for rapid growth. However, as mentioned above (point 3), the majority of SE seems to remain attached to the exterior of host cells and would not be growing rapidly.

[a] "Infection" is used to refer to the presence of SE in birds, whereas "contamination" is used to refer to the presence of SE in eggs.

Because of the greater amount of time before lay, we expect cells deposited earlier in egg formation (*Ey* or *Ev* or *Eac* contamination) would grow more than cells deposited later in egg formation (*Eaf* or *Es* or *Ep* contamination.) Moreover, there might be features of the albumen surrounding the yolk that would enhance SE growth, as suggested by data from Humphrey and Whitehead[6] indicating relative SE growth in albumen near the yolk is greater than that in albumen further from the yolk. However, others have reported "no general correlation" of growth of SE and other *Salmonella* strains in albumen incubated in the presence or absence of yolk.[7]

Contaminated eggs in experiments for which data are used extensively for determining values of parameters of exposure assessment models were inoculated with stationary phase SE culture preparations.[8;9] Predictive microbiology research suggests lag phase duration is influenced strongly by the condition of the inoculum. As discussed above, in the natural setting, before the egg is laid, SE within the egg could have experienced limited growth due to the internal reserve of nutrients within the SE cell. The biological reason for longer expected lags may be the need for physiological adjustment by SE from the nutrient-rich conditions of culture broth to the more stressful environment of egg albumen. Both dynamic pH and competition for free iron could be associated with longer lags in experimentally inoculated eggs than those for naturally infected hens. In either case, the lag phase durations are not known. State of knowledge assumptions were thus made concerning lag phase duration. In the risk assessment, the assumed lag times for cells in naturally contaminated eggs were assumed shorter than cells in experimentally contaminated eggs.

Yolk membrane breakdown (YMB)

After the egg is laid, through the process of osmosis, water seeps into and enlarges the yolk. This allows yolk material, particularly iron or other nutrients, to leave the yolk and become available to SE cells in the albumen and vitelline membrane. In time, the membrane weakens until a point where there is free exchange of material between albumen and yolk, upon which SE can grow rapidly. A primary question is how quickly this latter event, yolk membrane breakdown (YMB), occurs. The risk assessment model assumes YMB duration is short and models it at a specific time, during which the kinetics of *Salmonella* growth in yolk begin. States of knowledge assumptions were made for lag phase duration before SE begins to grow. The likelihood of YMB is dependent on temperature, levels of *Salmonella*, and location of *Salmonella* in the egg.

UNCERTAINTY IN THE RISK ASSESSMENTS

In these risk assessments, the term "variable" refers to a random variable that can take on different values for units of a well-defined population, where the frequency of the possible values within the population is determined by probability distributions. This definition is meant to include the degenerate case when there is only one possible value for the variable, usually determined by an assumption. For example, the lethality for a given process may be assumed constant for a given scenario of a risk assessment. The word "variability" for a variable then refers to the distribution of that variable over a well-defined population; to determine the variability of a variable is essentially the same as determining the variable's distribution.

"Parameters" refer to any object whose values or specific identities determine the characteristics, actions, or results of something in this case, the calculations of these risk assessments. Clearly then, "functions" and "populations" are parameters of a risk assessment, because the estimated risk depends upon the functions and populations considered; change the functions and populations, and the risk changes. When the true population is not known and data from other populations are available, then selecting the "population" to use introduces potential biases and thus introduces uncertainty. Examples of this occur when, for example, animal data are used to "represent" dose-response for humans, or, as in these risk assessments, spent hen data are used as proxy for commercial hens.

Parameters in these risk assessments always refer to entities (usually constant numbers) that affect the calculation of risk. For example, the parameter a could be the characterization that the variable x has a normal distribution with mean a: if the value of a changes, the distribution of x changes. In a risk assessment, values of parameters are assumed by some means, and the uncertainty of the assumed value reflects, in some sense, the degree of knowledge for the assumed value. A confusion of terminology arises when one wants to consider the variable x as a parameter; that is to say, treat it as a constant, and associate an uncertainty to it based on the distribution associated with x.

Typically, perfect knowledge of the "true" distribution of a variable is unachievable. Rather, the distributions are estimated by a variety of methods, depending upon available information. Two methods are germane to this discussion. In Method 1, probability distributions are estimated through a statistical analysis of data that are, in some well-defined way, "representative" of the population being studied. In Method 2, an assessment of anecdotal evidence based on perceptions of what is or might be, ideally from individuals who have had experience with the variable of concern, is used.

The assumption for Method 1 is that data are collected and represent, in a probabilistic fashion, a well defined population so that the values of the data are said to be "stochastic" realizations of some random variable. Statistical procedures can be applied to the data to derive estimates of the values of the relevant parameters that determine or characterize the distribution. For the purposes of these risk assessments, parameters that are used to characterize the distribution that are estimated from these data are termed stochastic parameters. Procedures have been devised to assess the accuracy of an estimated parameter from collected data. This assessment of the accuracy reflects the "uncertainty" of the estimated values of the parameters, referred to as "stochastic uncertainty."[10] Thus, when distributions for some variable are determined from data assumed probabilistically representative of some well-defined population, there is a clear distinction between what is termed variability and uncertainty; the predicate "stochastic" is attached to the parameters and the uncertainties associated with the estimation of possible values of the parameters. For example, if $:$ is a parameter whose value is statistically estimated from data, then $:$ is referred to as a stochastic parameter, and the uncertainty of its values is referred to as stochastic uncertainty.

Not all parameter values, however, can be estimated by Method 1. The determination or assumption for the values may be based on the opinions of experts, with the possible aid of anecdotal data. In this sense, the values determined for parameters depend strictly on one's state of knowledge;[10] thus this phrase is the predicate that is attached to parameters so determined. That is, a parameter is a "state of knowledge" parameter when its values are not determined from probabilistic representative data using statistical procedures of estimation. In such a situation, it is not possible to assess the accuracy of the assumed values in the same way that such an

assessment is made from data that are representative of a well-defined population. Rather, the assessment of accuracy is based on the same type of judgment that is used to derive the parameter's estimate. Consequently, there is no clear distinction between the assumed values and the assessment of the accuracy of the assumed values. In this situation, the uncertainty is termed "state of knowledge uncertainty;" a "likelihood" of the possible parameter values determined this way does not exist, at least in the same way it exists for assessment of stochastic parameters. Rather, the assessment and the assigned likelihoods are subjectively determined, dependent upon the beliefs of the people who made the evaluation. Consequently, in this situation, if possible, we have specified a set of values or a distribution we believe corresponds to the distribution of the variable. If distinct values are identified, reflecting the "uncertainty" of possible values for a parameter, then the risk assessment is computed separately for each of the distinct values, at least theoretically.

The following points of clarification that relate to these risk assessments are needed.

1) Some parameters for a distribution of a variable are stochastic and some are state of knowledge. In this case, the risk assessments assumed values for the state of knowledge parameter, and then estimated, conditional on these values, the values of the parameters, with their attendant uncertainty.

2) For some variables in these risk assessments, several functional forms were compared and, based on some measure of goodness of fit or other considerations, one of the functions was chosen to represent the distribution for that variable, or to describe a relationship between variables. However, in some cases, information was not available to make such comparisons; thus, one function was chosen, based on a common practice (e.g., a normal distribution) or as an accepted default (e.g., beta-Poisson for dose response). In one case, a clear selection could not be made, thus two functions were used; the risk assessments were performed using one function and then repeated using the other so to account for uncertainty of this parameter.

3) The population represented by data can also be thought of as a state of knowledge parameter, while parameters that define the distribution of variables associated with the population are stochastic. In other words, there exists data representing a population different from the population for which a distribution is desired. In all such cases, uncertainty associated with this parameter (the proxy population) regarding its relationship to the desired population was not accounted for. For example, the data from the USDA spent hen survey[11] does not represent commercial egg laying hens, so that the validity of using derived distributions from the survey to estimate distributions for commercial hens is based on judgment.

4) Uncertainty calculations were made with almost all parameters that characterize probability functions of functions that describe relationships between variables identified in the risk assessments. A primary exception is the distribution of the amount of egg consumed, for which standard errors of the computed percentiles are not included.

5) Stochastic uncertainties for estimated values of stochastic parameters are characterized by assigning "probability" distributions to possible values for the parameters. For the risk assessments, the distributions were determined by using asymptotic normal distributions used for approximating confidence regions for estimates of parameter values or by using a bootstrap procedure.

State of knowledge uncertainty implies a set of possible values for parameters that are determined by judgment. To determine the magnitude of this type of uncertainty for the outputs of the risk assessments, we defined subsets of assumed values from the set of possible values. For each subset, risk calculations were made that included the estimated probabilities of adverse events and other desired outputs of the risk assessments, together with attending stochastic uncertainty evaluations, expressed as confidence intervals. This can lead to an enormous number of calculations. One procedure to reduce the number of calculations is to choose values that represent the extremes of risk and the midpoint within the range of the possible values for the identified parameters (if possible), and compute the risks for these combinations. More involved calculations could be made with the purpose of finding a functional relationship between the possible values of the parameters and the risks. In effect, the output of these types of calculations can be thought of as multivariate, with fixed independent variables (representing the possible values of the state of knowledge parameters).

DATA REPRESENTATIVENESS

Critical to risk assessment is the representativeness of each individual input.[12] Data representativeness refers to how accurately data depict the true nature of things. Representativeness depends on factors such as how the experiments were designed and performed and whether they were repeatable. Throughout the risk assessment report, discussions of sample size, data variation, etc. are included when referring to specific data sets. In addition, sensitivity analyses were performed to quantifying the impact of input parameters on model predictions.

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