Research Note

Validation of a Predictive Model for Survival and Growth of *Salmonella* Typhimurium DT104 on Chicken Skin for Extrapolation to a Previous History of Frozen Storage[†]

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ABSTRACT

The U.S. Department of Agriculture's tertiary Pathogen Modeling Program (PMP) model for survival and growth of *Salmonella enterica* ser. Typhimurium definitive type 104 (DT104) on chicken skin stored for 0 to 8 h at 5 to 50°C was evaluated for its ability to predict survival and growth of the same organism on chicken skin after frozen storage for 6 days at −20°C. Experimental design and methods used to collect data for model development (dependent data) were the same as those used to collect data for survival and growth after frozen storage (independent data for extrapolation). This was done to provide a valid comparison of observed and predicted values. The model was classified as providing acceptable predictions of the test data when the proportion of residuals in an acceptable prediction zone (pAPZ) from −1 log (fail-safe) to 0.5 log (fail-dangerous) was ≥0.7. The pAPZ for dependent data, independent data for interpolation, and independent data for extrapolation to a new independent variable of previous frozen storage were all acceptable (pAPZ ≥ 0.7), with the exception of the pAPZ for dependent data at 50°C, where an unacceptable pAPZ of 0.625 was obtained. Although a majority of observed log counts were less than predicted log counts, indicating that frozen storage of chicken skin for 6 days at −20°C had injured some *Salmonella* Typhimurium DT104, the injury was not large enough to cause the tertiary PMP model to provide unacceptable predictions. Thus, it was concluded that the tertiary PMP model provided valid predictions of survival and growth of *Salmonella* Typhimurium DT104 on chicken skin that had a previous history of frozen storage for 6 days at −20°C. Additional research is needed to determine how broadly the model can be applied to other conditions of previous frozen storage.

The Predictive Microbiology Information Portal is a U.S. Department of Agriculture (USDA)–sponsored Web site (http://portal.arserrc.gov/) that seeks to educate large and small food companies about the proper use of predictive models for improving the microbiological safety of food. Included in the Web site is the USDA, Agricultural Research Service, Pathogen Modeling Program (PMP), which is a computer software application that contains predictive models for survival and growth of foodborne pathogens.

A recent addition to the PMP is a tertiary model that predicts the change in log counts of *Salmonella enterica* ser. Typhimurium definitive type 104 (DT104) on chicken skin as a function of time (0 to 8 h) and temperature (5 to 50°C) *(18)*. This model was validated with the acceptable prediction zone (APZ) method, which has been used to validate other predictive models *(1, 8, 10, 15, 19, 20, 22)*.

Validation of predictive models is an important step in their development, because it increases confidence in using them to help make food safety decisions. The APZ method systematically evaluates models for goodness of fit, interpolation, and extrapolation by using stated criteria for test data and model performance *(15, 19)*. Only when a model meets the test data and model performance criteria for goodness of fit and interpolation is it classified as validated. Evaluation of predictive models for extrapolation to new independent variables is done after validation and is important, because it saves time and money by identifying conditions for which new models are not needed *(14, 18, 19)*.

Although most chicken in the United States is sold fresh, it is common practice for consumers to purchase chicken in bulk and store some of it in home freezers. Survival of *Salmonella* on chicken during frozen storage depends on many factors such as rate of freezing, temperature of frozen storage, time of frozen storage, type of chicken meat, and strain of *Salmonella (2, 6)*. In general, there is an initial drop in the number of *Salmonella* during freezing, followed by a slower decline and plateau of survivors during extended frozen storage. Studies using nonselective and selective plating media indicate that under

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some conditions of frozen storage, *Salmonella* is injured *(5, 12)*, whereas under other conditions of frozen storage, *Salmonella* is not injured *(26)*. *Salmonella* injured by freezing exhibits reduced growth on exposure to favorable conditions, because additional time is needed to repair cellular damage caused by freezing before growth begins *(9)*.

The objective of the present study was to evaluate a tertiary model in the PMP for survival and growth of *Salmonella* Typhimurium DT104 on chicken skin stored for 0 to 8 h at 5 to 50°C *(18)* for its ability to extrapolate to a new independent variable of previous frozen storage. It was expected that frozen storage would reduce the number of *Salmonella* Typhimurium DT104 on chicken skin during subsequent exposure to cold stress, temperature abuse, and mild heating. Whether these effects would be large enough to cause the PMP model to provide unacceptable predictions of the log counts of *Salmonella* Typhimurium DT104 as a function of time (0 to 8 h) and temperature (5 to 50°C) was evaluated with the APZ method.

MATERIALS AND METHODS

General information. The tertiary PMP model evaluated in this study was created by combining secondary models for lag time and growth rate with a primary model *(14, 18)*. The tertiary PMP model predicts log counts of *Salmonella* Typhimurium DT104 on chicken skin as a function of time (0 to 8) and temperature (5 to 50°C) *(18)*. A full description of the tertiary PMP model and the methods used to collect data for development and validation (for interpolation) of the model has been published *(18)*, and the tertiary PMP model can be found on-line at http://pmp.arserrc.gov/PMPOnline.aspx?ModelID=28.

The description of experimental design and methods that follows is for the collection of test data for extrapolation of the tertiary PMP model *(18)* to a new independent variable of previous frozen storage for 6 days at −20°C. Experimental design and methods used to collect data for model development (dependent data) were the same as those used to collect data for survival and growth after frozen storage (independent data for extrapolation). This was done to provide a valid comparison of observed and predicted values discussed in a previous publication *(19)*.

Preparation of chicken. Fresh chicken thighs were purchased at local retail outlets. The skin was removed, spread on a plastic cutting board, frozen at −20°C for 15 min to facilitate cutting, and then cut into circular 2.14-cm^2 portions by using a no. 10 corkborer. Skin portions were placed back on top of the entire thigh minus bone in a 500-ml polycarbonate jar with a screw-cap lid. Chicken thighs prepared in this manner were stored overnight at 4°C before use in storage trials. The pH of chicken skin portions was 6.36 ± 0.19 (mean ± standard deviation [SD], $n = 16$), and the aerobic plate count at 30°C on brain heart infusion (BHI; BBL, BD, Sparks, MD) agar was 3.90 ± 2.31 log CFU per portion ($n = 23$).

Stock cultures. Viable cells of *S. enterica* ser. Typhimurium DT104 (ATCC 700408, American Type Culture Collection, Manassas, VA) were maintained in stock culture at −70°C in BHI broth that contained 15% (vol/vol) glycerol (Sigma, St. Louis, MO). This strain was used for model development *(18)* and validation due to its antibiotic resistance profile, which can be used for its differentiation from other microorganisms naturally present in food samples *(16)*.

Inoculation of chicken. Cultures of *Salmonella* Typhimurium DT104 for inoculation of chicken skin were initiated by inoculating 5 μl of stock culture into 5 ml of BHI broth in a 25-ml Erlenmeyer flask. The flask was sealed with a foam plug and incubated (Innova 4230 orbital shaking refrigerated incubator, New Brunswick Scientific, Edison, NJ) at 30°C and 150 rpm for 23 h to obtain stationary-phase cells for inoculation. Inoculation cultures, which contained 10.3 ± 0.04 log CFU/ml *Salmonella* Typhimurium DT104, were serially diluted (1:10) in buffered peptone water (BPW; Difco, BD) immediately before being used to inoculate chicken skin. Chicken-skin portions on top of thigh meat were spot inoculated on their surface with 5 μl of a 10^{-7} dilution of the inoculation culture. The number of *Salmonella* Typhimurium DT104 inoculated was calculated to be 0.98 ± 0.04 log CFU per portion ($n = 19$).

Frozen storage of chicken. After inoculation with *Salmonella* Typhimurium DT104, chicken thighs in polycarbonate jars were stored for 6 days at −20°C (Roper Refrigerator/Freezer, model RT14GDXAW00, Roper Appliance Group, Benton Harbor, MI). After frozen storage and before being used in storage trials, chicken thighs were thawed overnight at 4°C. This was done to standardize the protocol with that used in model development *(18)*, wherein chicken skin portions on thigh meat were held overnight at 4°C before inoculation and incubation at test temperatures.

Design of storage trials. To determine effects of previous frozen storage on survival and growth of *Salmonella* Typhimurium DT104 on chicken skin, thawed chicken thighs were stored at 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50°C for 0 to 8 h. A different batch of chicken thighs was used in each storage trial. The numbers of replicate storage trials conducted were one at 30°C, three at 40°C, and two at all other storage temperatures. A storage trial that was supposed to be conducted at 30°C was mistakenly conducted at 40°C.

Sampling of chicken. When sampling occurred every 2 h during the 8-h storage trial, two skin portions were processed at each sampling time, whereas only one skin portion was processed at each sampling time when sampling occurred at 1-h intervals during the 8-h storage trial. To recover *Salmonella* Typhimurium DT104 on chicken skin portions into BPW for subsequent enumeration, an individual skin portion was pulsified (Pulsifier model PUL 100, Microbiology International, Frederick, MD) for 1 min in 9 ml of BPW in a 207-ml capacity filter bag (Whirl-Pak, Nasco, Fort Atkinson, WI).

Pathogen enumeration. As described in a previous publication *(18)*, a combination of most-probable-number (MPN) and viable-count (CFU) methods were used to enumerate *Salmonella* Typhimurium DT104 on chicken samples. The tertiary PMP model evaluated in this study was used to produce a sampling schedule that included a determination of whether one or both enumeration methods should be applied to a sample. The plating medium used in the MPN and CFU methods was the same and contained multiple antibiotics to suppress the growth of the background microflora.

Model performance. The tertiary PMP model's *(18)* performance was evaluated with the APZ method *(15, 19)*. Residuals (observed log counts − predicted log counts) for individual prediction cases were calculated and were classified as acceptable when they fell within an APZ from −1 log (fail-safe) to 0.5 log (fail-dangerous). When the proportion of residuals in the APZ (pAPZ) for an individual survival or growth curve was ≥0.7,

TABLE 1. *Performance of the tertiary PMP model for survival and growth of* Salmonella *Typhimurium DT104 on chicken skin: evaluations for goodness of fit, interpolation, and extrapolation to a new independent variable of previous frozen storage*

Evaluation[a]	Type of data	Temp (°C)	Prediction cases		pAPZ[b]
			Acceptable	Total	
Goodness of fit	Dependent	5.0	16	19	0.842
		10.0	28	38	0.737
		15.0	32	40	0.800
		20.0	34	39	0.872
		25.0	43	50	0.860
		30.0	36	40	0.900
		35.0	38	40	0.950
		40.0	33	39	0.846
		45.0	29	39	0.744
		50.0	25	40	0.625
Interpolation	Independent	7.5	18	20	0.900
		12.5	14	20	0.700
		17.5	18	20	0.900
		22.5	18	20	0.900
		27.5	16	20	0.800
		32.5	16	20	0.800
		37.5	17	20	0.850
		42.5	16	19	0.842
		47.5	15	18	0.833
Extrapolation	Independent	5.0	14	19	0.737
		10.0	16	19	0.842
		15.0	14	19	0.737
		20.0	17	18	0.944
		25.0	16	18	0.889
		30.0	8	9	0.889
		35.0	17	19	0.895
		40.0	20	27	0.741
		45.0	14	16	0.875
		50.0	18	18	1.000

[a] Goodness of fit, evaluating the fit of the model to the data used in model development (dependent data); interpolation, evaluating the ability of the model to predict data not used in model development (independent data) but that were collected with combinations of independent variables (time and temperature) that were within the ranges of independent variables used to develop the model; extrapolation, evaluating the ability of the model to predict data not used in model development (independent data) but that were collected with combinations of independent variables (time and temperature) that were outside (e.g., previous frozen storage) the ranges of independent variables used to develop the model.

[b] Proportion of residuals in an acceptable prediction zone from −1 log (fail-safe) to 0.5 log (fail-dangerous).

the tertiary PMP model was classified as providing acceptable predictions of the test data.

Explanations for how the criteria for the APZ method were set can be found in previous publications (14, 15). Likewise, explanations for why the bias and accuracy factor method (21) was not used to evaluate performance of the tertiary PMP model can be found in previous publications (15, 18, 22). An explanation for why only the tertiary PMP model was evaluated and not also the secondary models for lag time and growth rate can be found in a previous publication (17).

Statistical analysis. Mean counts of *Salmonella* Typhimurium DT104 on chicken skin were compared with unpaired or paired Student's *t* tests (Prism, version 5.02 for Windows, GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Since publication of the tertiary PMP model evaluated in this study (17), the APZ method underwent a minor modification. Namely, it is now applied to individual survival and growth curves (19) rather than complete sets of data (15). This modification was made to better detect local prediction problems. Consequently, it was necessary to reevaluate the tertiary PMP model for goodness of fit and interpolation before evaluating it for extrapolation to a new independent variable of previous frozen storage.

The results of this reevaluation of tertiary PMP model performance (Table 1) revealed pAPZ for dependent data and pAPZ for independent data for interpolation that were all acceptable (i.e., ≥0.7) with one exception: the survival curve for dependent data at 50°C. Thus, the tertiary PMP model was validated for temperatures from 5 to 47.5°C. However, it was not validated for temperatures from >47.5 to 50°C. Thus, according to the test data criteria for the APZ method (19), it was not possible to completely validate the PMP model for extrapolation to a new independent variable.
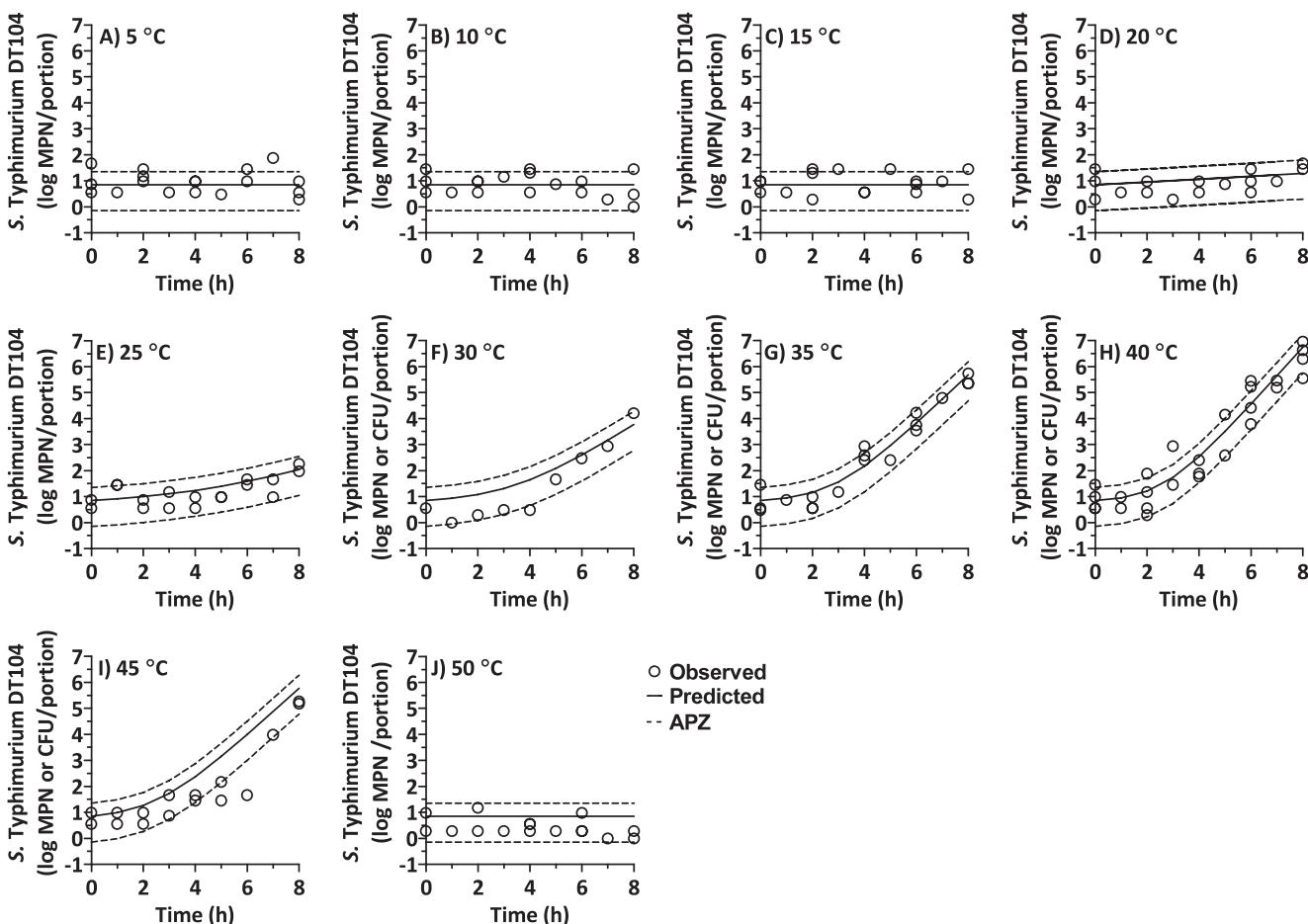
FIGURE 1. *Survival and growth of* Salmonella *Typhimurium DT104 on chicken skin after frozen storage for 6 days at* −20°C. *Symbols are observed values, solid lines are tertiary PMP predictions, and dashed lines are the boundaries of the acceptable prediction zone (APZ).*

Based on previous studies *(3, 5, 9)*, it was expected that frozen storage for 6 days at −20°C would kill some of the *Salmonella* Typhimurium DT104 inoculated onto chicken skin and injure others. Furthermore, it was expected that these effects of frozen storage would be observed as lower counts after frozen storage, cold stress, heat stress, and temperature abuse.

The mean count of *Salmonella* Typhimurium DT104 on chicken skin immediately after frozen storage was 0.85 ± 0.38 log MPN per portion (mean ± SD, $n = 27$); this value was not different ($P > 0.05$) from the mean count of *Salmonella* Typhimurium DT104 inoculated onto chicken skin before frozen storage, which was calculated to be 0.98 ± 0.04 log CFU per portion ($n = 19$). Thus, there was not any significant death of the pathogen on chicken skin during frozen storage for 6 days at −20°C.

Foster and Mead *(6)* reported that survival of *Salmonella* on chicken during frozen storage was greater at −20°C than at −5 or −2°C. Likewise, others found that survival of *Salmonella* in chicken drip *(13)* and on/in shrimp *(11)* is better at lower temperatures of frozen storage. Freezing at higher temperatures results in formation of larger ice crystals that produce more damage to microbial cells *(2)*.

Obafemi and Davies *(13)* reported that stationary-phase cells are more resistant to freezing than exponential-phase

cells, whereas Noda et al. *(11)* found that *Salmonella* inoculated onto the surface of shrimp are more resistant to freezing than those inoculated into flesh. In general, food protects *Salmonella* from lethal effects of freezing *(7)*, with a lower number of survivors observed when *Salmonella* is frozen in laboratory media than in food *(3, 4)*.

Thus, potential reasons that death of *Salmonella* Typhimurium DT104 was not observed during frozen storage in the current study are (i) frozen storage at −20°C rather than at a higher temperature, (ii) use of stationary-phase cells, (iii) surface inoculation of the organism, and (iv) use of chicken skin rather than laboratory broth.

The mean count of *Salmonella* Typhimurium DT104 on chicken skin after frozen storage and after subsequent storage for 1 to 8 h at 5, 10 or 15°C (Fig. 1A through 1C) was 0.89 ± 0.44 log MPN per portion ($n = 48$). This value was not different ($P > 0.05$) from the mean count of 0.98 ± 0.04 log CFU per portion of *Salmonella* Typhimurium DT104 inoculated onto chicken skin before frozen storage. Thus, previous frozen storage of the pathogen on chicken skin did not induce the type of injury that reduced counts on chicken skin during subsequent short-term (1 to 8 h) exposure to cold stress (5 to 15°C).

The mean count of *Salmonella* Typhimurium DT104 on chicken skin after frozen storage and subsequent incubation

for 1 to 8 h at 50°C (Fig. 1J) was 0.38 $\pm$ 0.32 log MPN per portion ($n = 15$). This value was lower ($P < 0.05$) than the mean count (0.98 $\pm$ 0.04 log CFU per portion) of the pathogen inoculated onto chicken skin before frozen storage. Thus, previous frozen storage of *Salmonella* Typhimurium DT104 on chicken skin for 6 days at −20°C induced the type of injury that reduced subsequent counts of the pathogen on chicken skin during short-term (1 to 8 h) exposure to 50°C, a mild heat stress. This finding is consistent with those of Smith et al. *(24),* who reported that frozen storage at −9°C reduced heat resistance of both exponential- and stationary-phase cells of *Salmonella* Typhimurium DT104 in ground beef. However, Singh et al. *(23)* found that frozen storage at −20°C for 1 to 120 days did not alter D_{62}- or D_{65}-values for *Salmonella* inoculated into irradiated ground beef.

The mean count of *Salmonella* Typhimurium DT104 predicted by the PMP model on chicken skin incubated for 3 to 8 h at 25 to 45°C (i.e., temperature abuse) was 3.32 $\pm$ 1.74 log MPN or CFU per portion ($n = 54$), whereas the mean count of *Salmonella* Typhimurium DT104 observed after frozen storage and subsequent incubation of chicken skin for 3 to 8 h at 25 to 45°C (Fig. 1E through 1I) was 3.04 $\pm$ 1.79 log MPN or CFU per portion ($n = 54$). Comparison of these two means by using a paired Student's *t* test to account for effects of time and temperature on absolute values of log counts indicated that previous frozen storage had caused a small (−0.29 log MPN or CFU per portion) but significant ($P < 0.05$) reduction in log counts of the pathogen. This finding agrees with that of Mackey and Derrick *(9),* who found that freezing of *Salmonella* Typhimurium in laboratory broth at −10°C reduced subsequent growth at 37°C. In contrast, White and Hall *(25)* reported that growth of *Salmonella* in beef or chicken during thawing at 20 or 27°C for 24 h was not affected by previous frozen storage at −18°C for 7 to 168 days.

Finally, ability of the tertiary PMP model *(17)* to extrapolate to a new independent variable of previous frozen storage was evaluated with the APZ method *(19).* Although a majority of observed log counts were below predicted log counts (Fig. 1), indicating that frozen storage for 6 days at −20°C had injured some *Salmonella* Typhimurium DT104 on chicken skin, the injury was not large enough to cause the tertiary PMP model to provide unacceptable predictions. In fact, the results of the APZ analysis (Table 1) indicated that the tertiary PMP model provided acceptable predictions (pAPZ $\geq$ 0.7) for all individual survival and growth curves obtained at 5 to 50°C after frozen storage for 6 days at −20°C. Thus, it was concluded that the tertiary PMP model provided valid predictions of the survival and growth of *Salmonella* Typhimurium DT104 on chicken skin that had a previous history of frozen storage for 6 days at −20°C.

Clearly, more research is needed to see how broadly the tertiary PMP model can be applied to chicken that has been frozen. Specifically, additional data are needed to evaluate extrapolation of the tertiary PMP model to other times and temperatures of frozen storage, as well as interactions of frozen storage conditions with other independent variables such as inoculum size, strain of *Salmonella,* and type of chicken meat (white, dark, or skin).

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