The Relationship between the Level of *Salmonella* Enteritidis and the Temperature at Which Eggs Have Been Held from the Day of Lay until the Day of Processing

Final Report - Revised

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SUMMARY

This study was undertaken to determine the relationship between the levels of inoculated *Salmonella* Enteritidis and simulated conditions at which eggs might be held from the day of lay until the day of processing.

Unprocessed chicken eggs (n = 1,920) of different sizes, with 480 of them being laid in winter, spring, summer, and fall, respectively were inoculated, in the albumin, with a five-strain mixture of *Salmonella* at 10^2 CFU/egg. Eggs not inoculated with *Salmonella* served as negative controls. Both the inoculated and the control eggs were stored at 4, 10, and 22°C (39.2, 50, and 71.6°F) for four weeks. The eggs were sampled twice a week, and at each sampling point, five eggs were assayed individually for populations of *Salmonella* and total aerobic counts. Samples tested negative for *Salmonella* were subjected to enrichment. The experiments were conducted with appropriate duplications and replications. None of the control eggs were found to be contaminated with *Salmonella*. Eggs laid in different seasons did not significantly impact the survival of the pathogen ($P > 0.05$). The populations of inoculated *Salmonella* were not significantly different in eggs stored at 4°C versus at 10°C ($P > 0.05$). However, eggs stored at 22°C had an overall *Salmonella* population that was 3.71 or 3.37 log higher than did the eggs stored at 4 and 10°C, respectively ($P > 0.05$). The overall mean *Salmonella* population at 22°C increased from the initial 2.12 to 3.36 Log CFU/ml after 2 weeks and to 7.84 Log CFU/ml after 3 weeks into the storage. A sharp increase in the populations of *Salmonella* occurred after the eggs were stored at 22°C for two to two and a half weeks. These results suggest that chicken eggs should not be stored at room temperature from the day of lay to the day of processing.

In a separate experiment, unprocessed chicken eggs (n = 960) of different sizes, with 240 of them being laid in winter, spring, summer, and fall, respectively were stored at 4, 10, and 22°C for four weeks. The eggs were sampled twice a week, and at each sampling point, the contents and shells of five eggs were assayed separately for *Salmonella*. A total of 14 presumptive colonies were isolated and none were confirmed to be *Salmonella*. Among the 14 isolates, 7 were *Proteus mirabilis*, 3 were *Enterobacter cloacae*, 2 were *Pseudomanas*, and 2 were *Escherichia coli*.

INTRODUCTION

Approximately 67-79 billion eggs are produced in the United States each year (Vogt, 1999; USDA, 2000; USDA Economic Resources Service. 2002). Of these, about 20 billion eggs are broken, pasteurized, and processed into liquid, dried or frozen egg products and the remaining are consumed as whole shell eggs (Vogt, 1999). Georgia is one of the ten largest egg-producing states in the country (American Egg Board, 2006) with 11.5 million layers and a total egg production of 3 billion. Georgia's egg industry is very important to the state's economy, accounting for a cash receipt of $368 million (Georgia Egg (Commission, 2006).

Approximately 1 in every 20,000 eggs produced in the U.S. is contaminated with *Salmonella*, especially with serotype *Salmonella* Enteritidis (Ebel and Schosser 2002). Contamination of eggs by *S*. Enteritidis is facilitated by the ability of the pathogen to cross the egg shells after the eggs are laid (Padron 1990; Chen et al., 1996; Miyamoto et al., 1998; Cox et al., 2000), and infect the reproductive systems of healthy hens and contaminate egg contents before egg shells are formed (Humphrey 1989; 1991; 1994). However, stringent procedures for cleaning and inspecting eggs implemented in the 1970s have made salmonellosis caused by external fecal contamination of shell eggs extremely rare (CDC, 1999). The initial level of *Salmonella* contamination in chicken eggs is about 20 cells within 3 weeks of laying (Humphrey et al., 1991; Humphrey and Whitehead, 1993). This level of contamination is expected to increase if eggs are not appropriately refrigerated after the eggs are laid. Previous research conducted in our laboratory, indicated that storage temperature had a significant influence on the quality and safety of eggs (Chen et al., 2005). When eggs were stored at 4^{o}C, their albumen had significantly less volume and less weight loss, as well as lower pH, which are indicators of slower egg aging process, in comparison to the egg albumen from storage at 10^{o} and 22^{o}C. Additionally, the vitelline membranes of refrigerated eggs were relatively stronger and required greater levels of energy to be ruptured. *Salmonella* flourished in eggs stored at 22^{o}C, even in the eggs with an initial level of contamination of 10^{2} CFU/egg. Storage at 4^{o} and 10^{o}C prevented *Salmonella* cells from growing in the eggs that had an initial inoculation level of 10^{2} or 10^{4} CFU/egg. When the initial level of inoculation reached 10^{6} CFU/egg however, the antimicrobial agents in the albumen and refrigeration storage were no longer capable of inhibiting the growth of *Salmonella*, and an outgrowth of the pathogen subsequently occurred.

Salmonella is the second most common cause of human enteric infections and affects approximately 1.4 million people each year in the United States with 500 ultimately resulting in death. The medical expenses, productivity losses, and number of deaths associated with illness caused by salmonellosis are estimated to be in the millions of dollars. In this study, we determined the relationship between the level of *Salmonella*, particularly *S*. Enteritidis, and the temperature at which eggs might be held. The project provides crucial information needed for implementation of the HACCP program in small and very small egg farms as well as egg processing facilities. It will help egg producers realize the importance of keeping eggs refrigerated, and provide regulatory agencies with baselines of *Salmonella* contamination in refrigerated as well as temperature-abused eggs before they enter egg processing facilities.

Part I: The Relationship between the Level of *Salmonella* Enteritidis and the Temperature at Which Eggs Are Held

MATERIALS AND METHODS

Eggs

Unprocessed chicken eggs (n = 1,920) of different sizes, with 480 of them being laid in January (winter season), March (spring season), July (summer season), and September (fall season), respectively, were obtained from a commercial processor in Georgia one day before the experiments. The eggs were transported under refrigerated conditions from the processor's farm to our laboratory in Griffin GA, and were stored at 4°C until use. Among the 480 eggs used in each season, 240 were used in the original experiment, with the remaining 240 in the replicate experiment.

Bacterial Strains and Growth Conditions

Five strains of *Salmonella* Enteritidis representing different phage types were used in the study. The *Salmonella* strains were retrieved from frozen stocks and cultivated on tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, MD) and bismuth sulfite agar (BSA; Difco, Becton Dickinson, Sparks, MD) at 37°C for 24 h. A single colony of each strain was aseptically transferred to tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) and incubated at 37°C for 16 h. A cocktail of a five-strain mixture was made by pooling equal aliquots of the five cultures. The mix was then appropriately diluted with buffered peptone water (BPW) to a concentration of 10^4 CFU/ml.

Inoculation and Storage

The chicken eggs described above were rinsed with deionized water, surface sterilized with 70% ethanol, and wiped with sterile paper towels. Holes on the egg shell were punched using sterile sharp-tip forceps. Fifty microliters of the *Salmonella* mixture at the concentration of 10^4 CFU/ml was inoculated into the egg albumen by inserting an 18 gauge needle on a 1 ml syringe at the 2 o'clock position in a path approximately parallel to the shell (Schoeni et al., 1995). The inoculated eggs were then stored at 4, 10, and 22°C for the period of four weeks to mimic the possible conditions under which eggs are held from the day of lay until the day of processing.

Salmonella Enumeration

The eggs were sampled twice a week and at each sampling point five eggs were taken from the storage and assayed individually for the population of *Salmonella* Enteritidis and total aerobic counts. The experiments have appropriate duplications and replications. To ensure there was no prior contamination, negative control eggs were sampled simultaneously with the inoculated eggs at each sampling point. Eggs that were cracked during transportation and handling were discarded. On each sampling day, eggs were withdrawn from the storage, swabbed with 70% ethanol, and cracked with a sterile spatula in a sterile plastic bag. The contents of the eggs were homogenized using a stomacher (Seward, UK) for 2 min, and serially diluted in BPW.

Appropriate dilutions were plated in duplicate on BSA for the enumeration of *Salmonella* and on TSA for total aerobic counts. The inoculated plates were incubated at 37°C for 24 h and colonies appearing on the plates were counted using an automatic colony counter.

Enrichment

The samples that tested negative on the BSA plates were subjected to the enrichment procedures for *Salmonella*. Twenty-five ml of the egg contents were transferred in to 225 ml of BPW, and the samples were incubated for 24 h at 37°C. One milliliter of the sample was then transferred into 9 ml of Rappaport Vassiliadis broth (Difco, Becton Dickinson), and incubated at 41.5°C for 24 h. A loop full of the enriched cultures was then streaked on BSA plates, and appearing colonies were confirmed by biochemical and agglutination tests.

Statistical Analysis

Randomized complete block design was used in this study with three different storage temperatures (4, 10, and 22°C) and five different storage times (0, 1, 2, 3 and 4 weeks). Data was analyzed with the general linear model procedure using Statistical Analysis Software (SAS 9.1, SAS Institute, Inc., Cary, N.C.) at a 95% confidence level.

RESULTS

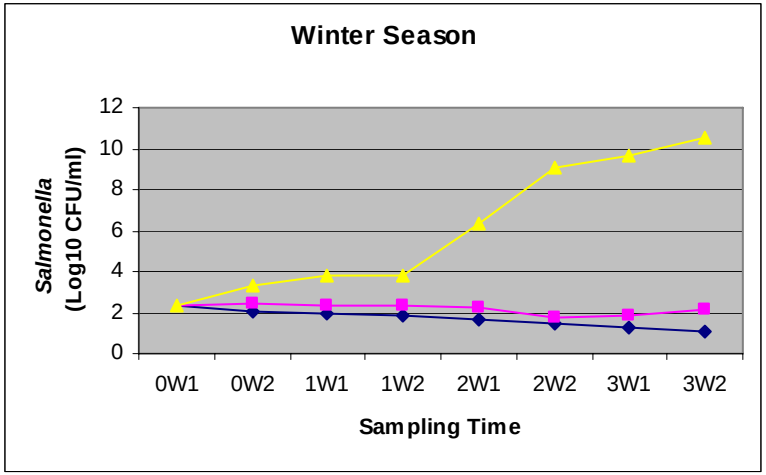
None of the control eggs were found to be contaminated with *S.* Enteritidis. The populations of total aerobes and the inoculated *Salmonella* were not significantly different in eggs that were laid in different seasons ($P > 0.05$; Table 1). Figure 1 shows the influence of storage temperature and storage time on the fate of artificially inoculated *Salmonella* in eggs laid in January (Figure 1A), March (Figure 1B), July (Figure 1C), and September (Figure 1D), respectively, whereas the influence of storage conditions on the population of total aerobes is shown in Figure 2. The populations of *Salmonella* decreased slightly during the four week storage period at 4°C. Only in 2 out of the 80 eggs laid in January and stored at 4^{o}C, did the populations of *Salmonella* fall below the detection levels (1 CFU/ml) however, these 2 eggs were later confirmed positive for *Salmonella* after the enrichment. At 10°C there was no significant increase or decrease in the numbers of the *Salmonella* cells in artificially contaminated eggs. Only 1 out of the 80 eggs laid in January and stored at 10^{o}C tested negative for *Salmonella* by direct plating. Viable *Salmonella* cells were however, recovered from the eggs after the enrichment procedure. The populations of inoculated *Salmonella* were not significantly different in eggs stored at 4^{o}C versus 10^{o}C ($P > 0.05$; Table 1). However, eggs stored at 22^{o}C had significantly higher populations of *Salmonella*. At this temperature, the populations of *Salmonella* in the eggs increased from the initial 2.12 Log_{10} CFU/ml to 3.36 Log_{10} CFU/ml after 2 weeks and 7.84 Log_{10} CFU/ml after 3 weeks into the storage. A sharp increase in the populations of *Salmonella* occurred after the eggs were stored at 22^{o}C for two to two and a half weeks. A sharp increase in the populations of *Salmonella* and total aerobes occurred after the eggs were stored at 22°C for two or two and a half weeks. These results suggest that chicken eggs should not be stored at this temperature from the day of lay to the day of processing.

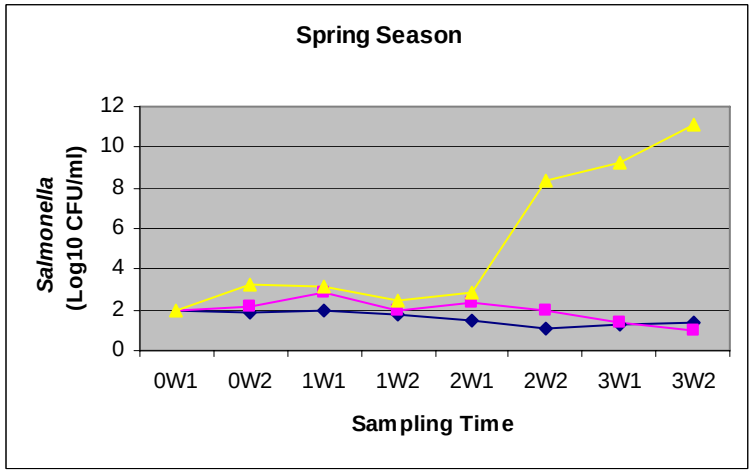
Table 1. Overall mean populations of *Salmonella* and total aerobic bacteria and results of statistical analysis

	Microbiological Media	
	TSA	BSA
Season		
Winter	3.49a	3.34a
Summer	3.29a	3.29a
Spring	3.02a	2.95a
Fall	2.89a	2.70a
Storage Temperature (^{o}C)		
22	5.40a	5.43a
10	2.28b	2.06b
4	1.84b	1.72b
Storage Time		
3W2	4.63a	4.24a
3W1	4.26ab	3.96a
2W2	3.48bc	.73ab
2W1	2.89cd	2.88bc
1W1	2.68cd	2.60c
0W2	2.67cd	2.57c
1W2	2.62cd	2.47c
0W1	2.15d	2.12c

*: Means followed by the same letters, in the same column and within the category of sampling seasons, storage temperatures, or lengths of storage time, are not significantly different.

A





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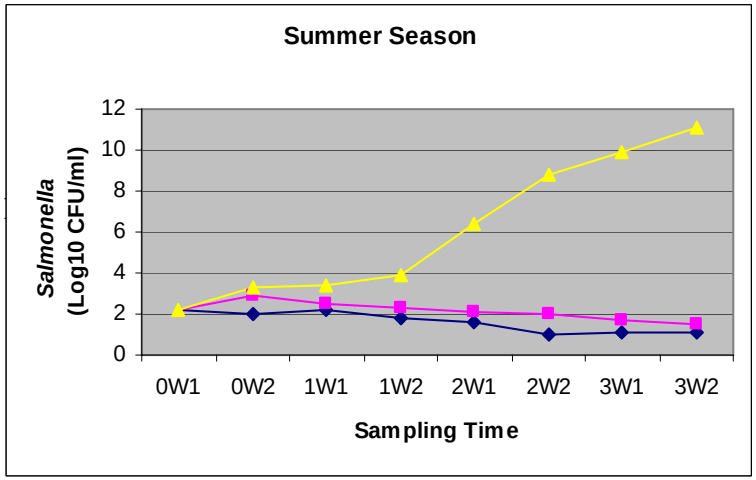

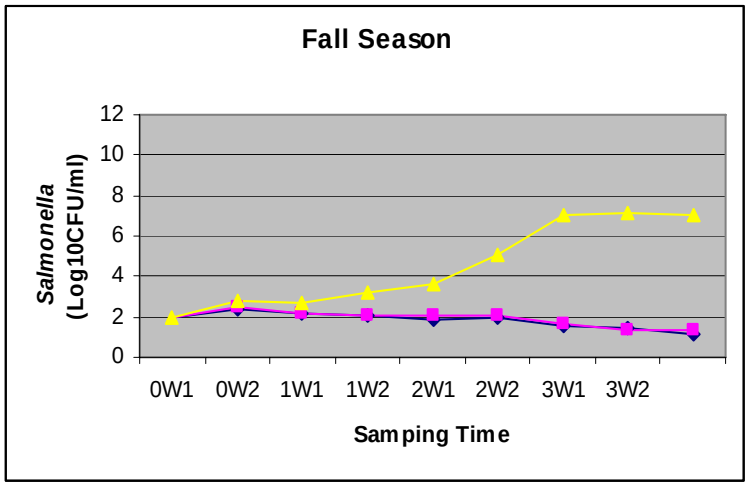

Figure 1. Populations of *Salmonella* Enteritidis in artificially inoculated chicken eggs stored for four weeks at 4°C (♦), 10°C (■), and 22°C (▲), respectively. A: Data from the winter trial (n = 480), B: Data from the spring trial (n = 480), C: Data from the summer trial (n = 480), and D: Data from the fall trial (n = 480). Sampling times included 0 - 3 weeks (0W - 3W) with two samplings each week (sampling 1 - 2)

D

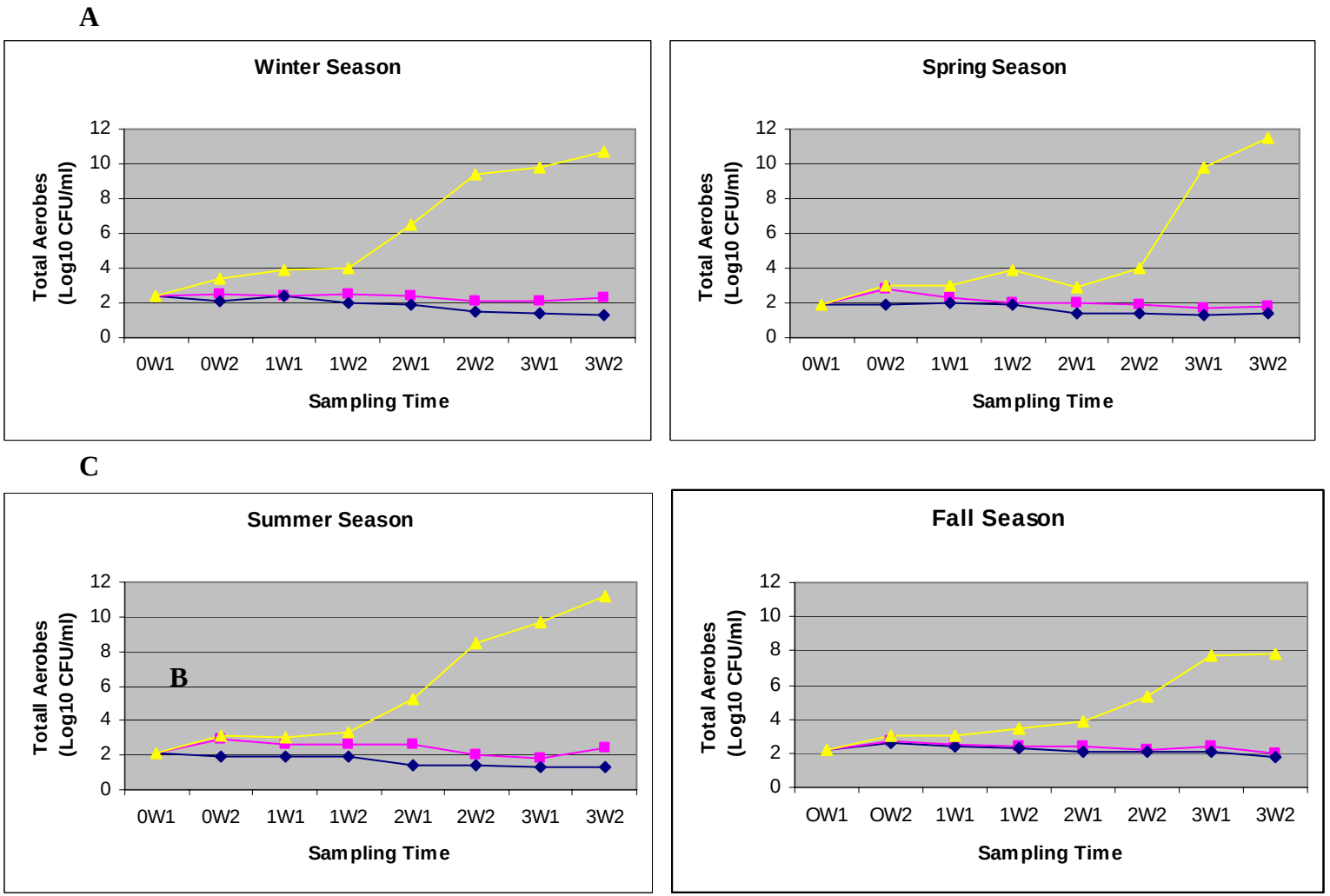

Figure 2. Populations of total aerobes in artificially inoculated chicken eggs stored for four weeks at 4°C (♦), 10°C (■), and 22°C (▲), respectively. A: Data from the winter trial (n = 480); B: Data from the spring trial (n = 480); C: Data from the summer trial (n = 480); and D: Data from the fall trial (n = 480). Sampling times included 0 - 3 weeks (0W - 3W) with two samplings each week (sampling 1-2)

D

Part II: Isolation of *Salmonella* from Chicken Eggs Stored at Different Temperatures

TASK SUMMARY

A total of 960 fresh chicken eggs were sampled for *Salmonella* over one year period, with 240 eggs being sampled in January, April, July, and September, respectively. The contents as well as the shells of all eggs were separated, sampled, and enriched for *Salmonella*.

MATERIALS AND METHODS

Source of Eggs

During each of the four seasons, a total of 240 freshly laid, unprocessed chicken eggs were obtained, on 2 separate occasions, from a commercial egg processor in Georgia. The initial trial of the experiment was conducted with the first group of eggs (n = 120), then repeated with the second group of eggs (n = 120). Both groups of eggs were transported to our laboratory under refrigerated conditions one day prior to the experiments, and stored at 4, 10, and 22^{o}C, respectively with 40 eggs at each temperature in each trial.

Egg Sampling

In each trial, egg sampling was performed twice per week during a four week-storage period at 4, 10, and 22^{o}C, with five eggs being withdrawn from each storage temperature at each sampling point. On the day of sampling, each egg was cleaned with running tap water, disinfected with 70% (v/v) of ethyl alcohol, and dried with sterile paper towels. After cleaning and disinfecting the surface, each egg was aseptically cracked open with a sterile metal scoop. The egg was separated into shell and liquid egg, and sampled separately. The liquid egg contents were placed in a sterile Whirl-Pak bag, and homogenized in a Stomacher Lab Blender (Seward Lab System, UK) at normal speed for 2 min.

Shell Sampling

The shells of the eggs were transferred to a different sterile Whirl-Pak bag containing 25 ml of lactose broth. The shells and egg membranes were gently crushed with a pair of gloved hands for approximately 30 seconds (Berrang et al., 1991; Chang 2000).

Direct Plating

One ml of each homogenized liquid egg sample and shell-rinsing broth was inoculated separately onto bismuth sulfate agar (BSA) plates. The inoculated plates were incubated at 37^{o}C for 24 h. Typical colonies, if any, were selected from the BSA plate and confirmed by biochemical and serological tests.

Salmonella Enrichment

After homogenization, 25 ml of liquid eggs or shell-rinsing broth were transferred to a sterile bag which contained 225 ml of lactose broth. The samples were allowed to stand at room temperature for one hour. The pH of the pre-enrichment mixtures was adjusted to 6.8 ± 0.2 with 0.1 N HCl or 0.1 N NaOH and 2.25 ml of Triton X-100 (Fisher Scientific, Fair Lawn, NJ) was then added. The pre-enrichments were conducted at 37^{o}C for 24 h, and following incubation one ml of the pre-enriched cultures was transferred to 10 ml of tetrathionate (TT) broth and 0.1 ml of the pre-enriched cultures was transferred to 10 ml of Rappaport-Vassiliadis (RV) broth. The inoculated broth was incubated at 42^{o}C for 24 h after which, one ml of each broth culture was streaked separately onto BSA plates which were incubated at 37^{o}C for 24 h. Presumptive *Salmonella* colonies were selected and grown on MacConkey agar at 37^{o}C for 24 h. Selected colorless colonies on MacConkey agar were then inoculated into triple sugar iron agar (TSI) and lysine iron agar (LIA) for biochemical confirmation. Isolates from presumptive positive TSI slants were confirmed using agglutination assays. All dehydrated media used in the study were obtained from Difco Laboratories.

RESULTS

Out of the 960 liquid egg samples and 960 egg shells sampled in the project a total of 14 presumptive *Salmonella* colonies were recovered from 4 eggs and 6 samples (Table 2). However, none of the colonies were confirmed to be *Salmonella*. Among the 14 isolates, 7 were *Proteus mirabilis*, 3 were *Enterobacter cloacae*, 2 were *Pseudomanas* spp., and 2 were *Escherichia coli*.

PROJECT DELIVERABLES

The results of the research will be posted on the website of the Food Product Innovation and Commercialization Center at The University of Georgia, and perhaps the website of American Egg Board and Egg Nutrition Center. An abstract has been submitted to the Institute of Food Technologist (IFT) and, upon acceptance, a poster presentation will be made at the annual meeting of IFT in July 2007. After which a manuscript will be submitted to the *Journal of Food Protection* for publication in 2007-2008.

REFERENCES

American Egg Board. 2006. Egg industry fact sheet – April (2006.) http://www.aeb.org/Industry/Facts/FactsSheet.htm.

Berrang, M. E., N. A. Cox, J. S. Bailey, and L. C. Blankenship. 1991. Methods for inoculation and recovery of *Salmonella* from chicken eggs. Poult. Sci. 70:2267-70.

CDC. 1999. Prevalence of *Salmonella* spp. and *Escherichia coli* on U.S. livestock operations.

Chang, Y. H. 2000. Prevalence of *Salmonella* spp. in poultry broilers and shell eggs in Korea. J. Food Prot. 63:655-8.

Chen, J., R. Clarke, and M.W. Griffiths. 1996. Penetration of *Salmonella enteritidis* through whole-shell eggs and survival of the bacteria in whole-liquid egg during refrigeration storage. J. Food Prot. 9:915-921.

Chen, J., H. S. Thesmar, and W. L. Kerr. 2005. The outgrowth of salmonellae and the physical property of albumin and vitelline membrane as influenced by egg storage conditions. J. Food Prot. 68:2553-2558.

Cox, N. A., J. S. Bailey, R. J. Buhr, and M. E. Berrang. 2000. Bactericidal treatment of hatching eggs. IV. Hydrogen peroxide applied with vacuum and a surfactant to eliminate *Salmonella* from hatching eggs. J. Appl. Poult. Res. 9:530-534.

Ebel, E. and W. Schosser. 2002. Estimating the annual fraction of eggs contaminated with *Salmonella enteritidis* in the United States. Int. J. Food Microbiol. 61:51-62.

Georgia Egg Commission. 2006. Governor Purdue proclaims egg month. http://www.georgiaeggs.org/pages/news.html.

Humphrey, T. J., J. G. Cruickshank, B. Rowe, and S. Hopper. 1989. *Salmonella enteritidis* phage type 4 from the contents of intact eggs: a study involving naturally infected hens. Epidemiol. Infect. 103:415-423.

Humphrey, T. J., A. Whitehead, A. H. L. Gawler, A. Henley, and B. Rowe. 1991. Numbers of *Salmonella enteritidis* in the contents of naturally contaminated hens' eggs. Epidemiol. Infect. 106:489-496.

Humphrey, T. J., and A. Whitehead. 1993. Egg age and the growth of *Salmonella enteritidis* PT4 in egg contents. Epidemiol. Infect. 11:209-219.

Humphrey, T. J., K. W. Martin, and A. Whitehead. 1994. Contamination of hands and work surfaces with *Salmonella enteritidis* PT4 during the preparation of egg dishes. Epidemiol. Infect. 106:489-496.

Miyamoto, T., K. Sasai, E. Baba, T. Horie, and T. Fukata. 1998. Changes in microflora of the cloaca and oviduct of hens after intracloacal or intravaginal inoculated with *Salmonella enteriridis*. Avian Dis. 42:536-544.

Padron, N. A. 1990. *Salmonella typhimurium* penetration through the eggshell of hatching eggs. Avian Dis. 34:263-465.

Schoeni, L. J., K. A. Glass, J. L. Mcdermott, A. C. L. Wong. 1995. Growth and penetration of *Salmonella heidelberg* and *Salmonella typhimurium* in eggs. Int. J. Food Microbiol. 24:385-386.

USDA. 2000. Egg production and distribution. http://www.aeb.org/eii/facts/us-prod.html.

USDA Economic Resources Service. 2002. Brief room: poultry and eggs. http://www.ers.usda.gov/Briefing/Poultry/Data/data.htm.

Vogt, D. U. 1999. Congressional research service report for Congress, Federal regulatory structure for egg safety: fact sheet. http://www.cnie.org/NLE/ag-90.html.