ABSTRACT

Title of Dissertation: EPIDEMIOLOGY OF SALMONELLA CONTAMINATION OF POULTRY MEAT PRODUCTS: KNOWLEDGE GAPS IN THE FARM TO STORE PRODUCTS

Maung San Myint, Doctor of Philosophy, 2004

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The ultimate goal of controlling foodborne hazards is to reduce the risk of disease to consumers, and the economic burden related to foodborne illness. A literature review identified four areas of insufficient data on the epidemiology of *Salmonella*. A cross-sectional study was used to estimate prevalence of enteric bacterial contamination of plant-based animal feed and milk replacer from Maryland and Northern Virginia. All samples were negative for *Campylobacter*; 0.6% were positive for *Salmonella*; 5.7 % for *E.coli*; and 50.6% for *Enterococcus*. Samples purchased in summer of 2002 were 38 times more likely to be contaminated with *Enterococcus* than samples purchased in

winter of 2002 (p-value<0.001). *Enterococcus* positive samples were 8 times more likely to be *E.coli* positive than *Enterococcus* negative samples.

Another cross-sectional study was used to assess the association between the pattern of airflow and the distribution of fecal coliforms and *Salmonella* in commercial chicken litter. At moderate relative humidity (about 50%), there was a significant association between regions of reduced airflow and increased coliform and *Salmonella* contamination within a poultry house.

An analysis of a PCR technique to validate sensitivity and specificity relative to culture techniques for detecting *Salmonella* contamination in retail poultry meat was conducted. When only BPW pre-enrichment was used, the PCR test had a sensitivity of 85%. This increased to 89 - 100% when BPW pre-enrichment was followed by selective enrichment with RV or TT-H broth, respectively when conventional culture is the gold standard. A minimum of 12 hours pre-enrichment and 100 cfu was necessary to achieve 100% sensitivity with PCR.

Random poultry meat samples from 10 retail grocery outlets in Maryland were collected in the final cross-sectional study. Overall *Salmonella* prevalence in poultry meat products was 23% (C.I 15.16 - 30.86). Integrator brand ground chicken meat had an increased *Salmonella* prevalence compared to non-ground meat products; however this difference was not significant (p=0.0533). Store brand non-ground chicken meat products were 18 times more likely to be contaminated with *Salmonella* than integrator brands (C.I. 5.41-61.26).

EPIDEMIOLOGY OF SALMONELLA CONTAMINATION OF POULTRY MEAT PRODUCTS: KNOWLEDGE GAPS IN THE FARM TO STORE PRODUCTS

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Foreword

As an advisor to Maung San Myint I have had the honor of helping to guide a promising new researcher through the completion of his doctoral dissertation and beginning a career as an epidemiologist. Those that have survived veterinary school and been admitted to a PhD program have already demonstrated their intellectual capacity and potential for success. However the ability to complete a PhD also depends on the three D's – drive, determination, and dedication. San has demonstrated an abundance of these.

This dissertation is a testament to San's ability to overcome obstacles, face challenges, and continue to strive toward a goal in the face of disappointment. In spite of all of these limitations, San's research has made a significant contribution to the body of knowledge on the epidemiology of *Salmonella* contamination of poultry products. The studies that comprise this dissertation have identified risk factors for *Salmonella* contamination from on-farm production to retail distribution and a standard protocol for detecting *Salmonell*a has been recommended and validated for use in retail poultry meat products.

I feel proud to call him a colleague and privileged to call him a friend. Best wishes for the future!

Dr. Yvette J. Johnson

DVM, MS, Ph.D

Dedication

To my parents, my wife Thet and two beloved daughters Irisa and Melissa
for their tireless support and understanding during my study

Acknowledgments

I am greatly indebted to Dr. Yvette J. Johnson for the valuable help and encouragement she gave me throughout my study and work at Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) at College Park, Maryland and for her unfailing interest, stimulating supervision, constructive criticism, which made the completion of this work possible. I extend my gratitude to my advisory members, Drs. Nathaniel L. Tablante, Estelle Russek-Cohen, Robert A. Heckert and Joseph C. Paige for their help and advice. Also, I would like to thank Dr. Edward T. Mallinson for offering his time and microbiological expertise, Dr. Ana Baya and Dr. Daniel Baustista for their help in microbial isolation and identification from animal feed samples and Mr. Russell Miller for in-depth knowledge about *Salmonella* and for giving help and encouragement.

My thanks and gratitude also extends to Dr. Siba K. Samal (Chair, Department of Veterinary Medicine-VMRCVM, College Park, MD) for his support to finish my studies successfully and for allowing me to use his laboratory. I also wish to express my thanks and gratitude to all of my colleagues from VMRCVM who helped, critiqued, and assisted me during this project. Also, I would like to thank Solar Biologicals, Inc. and Becton and Dickinson & Co. for providing drag swabs and MM media for my research.

Finally, I am indebted to my wife *Thet*, my daughters *Irisa* and *Melissa* and my family for their understanding, support, and love which allowed me to finish my study successfully

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1.1 Rationale

Salmonella species are responsible for a variety of acute and chronic diseases in both poultry and humans. Infected poultry products are among the most important sources for foodborne outbreaks in humans. Isolations of *Salmonella* are reported more often from poultry and poultry products than from any other animal species. The genus *Salmonella* of the family *Enterobacteriaceae* consists of more than 2300 serologically distinguishable variants. According to the Centers for Disease Control and Prevention, *Salmonella* alone affects about 1.4 million people each year in the United States with about 16,000 hospitalizations and more than 500 deaths annually. In 1996, the USDA, Economic Research Service estimated that the total costs for medical care and lost productivity, resulting from foodborne *Salmonella* infections of humans was between 0.6 – 3.5 billion dollars annually. Other costs associated with salmonellae include various direct expenses producers face as a consequence of *Salmonella* infection in their flocks. Control measures such as biosecurity practices, cleaning and disinfecting of facilities, rodent control programs, vaccination, and testing all can significantly increase production costs. Moreover, *Salmonella* contamination of food products can significantly reduce consumer demand and affect producer profits.

1.2 Problem Statement

Commercial poultry constitute one of the largest and most important reservoirs of paratyphoid (PT) salmonellae that can be introduced into the human food supply. Controlling paratyphoid (PT) infections has thus become an important objective for the

poultry industry from both public health and economic perspectives. There has been extensive research related to food safety in every aspect of production, transportation, processing, storage, and food preparation. However, in spite of the quantity of information available, there are still gaps in our knowledge of food safety, especially if we consider the entire farm-to-fork production model. There are still risks that have yet to be clearly identified or quantified. There are also tools that need to be enhanced or developed such as production and storage techniques for *Salmonella*-free animal feed, rapid and reliable *Salmonella* detection methods and effective on-farm environmental control measures.

1.3 Goals and Objectives

The overall goals of this research are to enhance food safety and fill some of the knowledge gaps in the epidemiology of *Salmonella* from the farm-to-retail store poultry production continuum. Several specific objectives have been identified to achieve these goals:

- To estimate the prevalence of enteric microbes (*E.coli, Salmonella, Campylobacter* and *Enterococcus spp.*) in vegetable protein for animal feed and milk replacer.
- To evaluate the relationship between airflow patterns and the distribution of *Salmonella* and fecal coliforms (FC) in poultry litter.
- To estimate the effect of different enrichment protocols on the sensitivity and specificity, and detection limit of a PCR method to detect naturally contaminated meat samples.

- To identify risk factors and estimate the *Salmonella* prevalence in chicken meat products from retail grocery stores in Maryland.

1.4 Hypotheses

Chapter 2

- The prevalence of *Salmonella, E.coli, Campylobacter,* and *Enterococcus* in retail plant-based animal feed and milk-replacer is greater than or equal to 10%.
- There is no significant difference in the distribution of *Salmonella, E.coli, Campylobacter*, and *Enterococcus* in retail plant-based animal feed and milk-replacer during different seasons.

Chapter 3

- Litter samples from regions of reduced airflow within a poultry house are associated with increased *Salmonella* and *E.coli* bacterial loads.

- Regions within a poultry house with below median airflow are at increased risk for being *Salmonella* positive and have greater *Salmonella* and *E.coli* loads than regions within a house with above median airflow.

Chapter 4

- Pre-enrichment causes no significant difference in sensitivity and specificity of PCR when compared to culture for the detection of *Salmonella* in naturally contaminated retail poultry meat.
- PCR and conventional culture have a concordance rate of greater than or equal to 95% when applied in the testing of naturally contaminated retail poultry meat.

Chapter 5

- There is no difference in the risk of *Salmonella* contamination of store brand poultry meat products than integrator brand poultry meat products.

- Ground chicken meat products have a higher risk of *Salmonella* contamination.

1.5 Overview

Each chapter in this dissertation is written in a format suitable for publication independently. Thus, each chapter has an abstract, introduction, hypothesis, methods, results and discussion (Chapter 1 is a literature review and does not have hypotheses or methods). The first chapter defines the scope of the economic problem by examining the effects of salmonellosis in an epidemiological context. This chapter reviews the current literature focusing on the first three phases of the poultry production continuum: on-farm production, processing by the poultry integrator, retail distribution and sale. The final phase, in-home handling, preparation and eating habits by the consumer, is beyond the scope of this dissertation. The literature on each of the three phases of production are evaluated with respect to the current knowledge regarding the prevalence of *Salmonella* contamination at each stage of production, identification of risk factors for *Salmonella* contamination at each stage, techniques for the rapid detection of *Salmonella* contamination, and the efficacy of current prevention and control measures for reducing the level of *Salmonella* contamination at each production phase. The review highlights areas in the literature where data are lacking or inconsistent and provides a comprehensive foundation for further investigation.

Based upon the extensive literature review, four areas of study have been identified as gaps in the body of knowledge on the epidemiology of *Salmonella* contamination of poultry products:

1) The role of plant-based feed as a potential source of microbial contamination.

2) Increased airflow as a means of reducing microbial contamination within the poultry houses.

3) The validity of new PCR-based techniques for detection of naturally contaminated field samples.

4) The prevalence of *Salmonella* contamination of poultry products at the retail grocery outlet and the risk factors for such contamination.

These 4 topics form the basis of the research for this dissertation. Chapters 2, 3, and 5 are cross-sectional studies. The cross-sectional study design has the benefit of providing a snapshot of a defined population. It estimates the scope of the problem in a representative sample of the population and identifies associated risk factors. While the nature of cause and effect relationships cannot be established, the cross-sectional design is a useful tool for generating hypotheses to test in future prospective studies.

The second chapter is a cross-sectional study designed to estimate prevalence of enteric bacterial contamination in plant-based animal feed products and milk replacer obtained from several retail outlets in Maryland and northern Virginia. Feed has been identified as an important source of *Salmonella* contamination of livestock environments. While there are several studies documenting the contamination of animal-by product based-feed with various enteric bacteria including *Salmonella*, there has been limited

research documenting the role of plant-based feed and milk replacers may have on the epidemiology of *Salmonella* in livestock.

The third chapter also uses a cross-sectional study approach to assess the association between airflow in commercial broiler houses and the prevalence of enteric pathogens in poultry house litter. The role of environmental moisture as a risk factor for *Salmonella* contamination has been documented in several studies. Increased airflow within the poultry house as a means of reducing litter moisture may be a simple, cost-effective means of reducing litter contamination by enteric bacteria.

The development of sensitive, specific, and rapid tests for the detection of *Salmonella* contamination is essential to control efforts. The fourth chapter evaluates the sensitivity and specificity of a PCR based diagnostic test for detection of *Salmonella* contamination of poultry products, compared to conventional culture techniques.

The retail grocery outlet is the last phase of the production continuum before the product is transferred to the consumer. If proper techniques are not in place to ensure the wholesomeness and safety of the poultry product at this level, then all the control measures employed previously will be for naught. Despite the importance of this phase of production, few studies detail the prevalence of *Salmonella* contamination of poultry products at the retail outlet or identify risk factors for contamination. The fifth chapter of this dissertation is a cross-sectional study of retail poultry meat in Maryland. The distribution of serotypes and risk factors for contamination are investigated.

The dissertation concludes with an overall summary and recommendations for future research.

2. Chapter I: Epidemiology of *Salmonella* Contamination of Poultry Products

2.1 Introduction

Salmonellae remain among the leading sources of food-borne illness throughout the world, even though many other pathogens have recently received considerable media attention. Infections of domestic poultry with salmonellae are expensive both for the poultry industry and for society as a whole. The costs associated with *Salmonella* paratyphoid contamination (PT) of poultry fall into two broad categories. The first category is related to expenses associated with human illnesses caused by the consumption of contaminated poultry products. The total combined costs associated with medical care and lost productivity resulting from foodborne non-typhoidal *Salmonella* infections of humans in the United States alone have been estimated at up to $ 3.5 billion for 1993.[151] The US annual salmonellosis burden was recently estimated to be in the order of 1.5 million cases (including over 580 deaths), 95% of these cases were attributed to foodborne infection[115]. The second category of costs associated with salmonellae in poultry involves various direct expenses producers face as a consequence of *Salmonella* infections in their flocks.

Reducing the risk of human salmonellosis from consumption of poultry products requires addressing three issues:

1) Increased understanding of the epidemiology of the serotypes of *Salmonella* associated with poultry that are responsible for foodborne disease in humans;

2) Development and validation of sensitive, specific and rapid detection strategies to identify sources of contamination during on-farm production, integrator processing, retail distribution, and consumer storage, preparation and consumption; and

3) Development of cost-effective prevention and control strategies for each stage of the production continuum.

The objective of this paper is to review the current literature on the epidemiology of *Salmonella* contamination of poultry, current advances in the detection of *Salmonella*, and to discuss the various *Salmonella* control strategies. The review highlights knowledge gaps in the current literature and provides a comprehensive foundation for further investigation.

Motile *Salmonella* serotypes (PT salmonellae) other than those in the *S. arizonae* subgenus can infect a very wide variety of hosts including humans, in some instances resulting in relatively asymptomatic intestinal carriage and in other instances producing clinical disease. PT salmonellae have been the subject of intensified interest as agents of foodborne disease transmission to humans. Controlling PT infections has become an important issue for the poultry industry from both public health and economic perspectives. The ultimate goal of controlling foodborne hazards is to reduce the risk of disease to consumers, and reduce the economic burden related to foodborne illness. The 'farm-to-fork' production continuum has emerged as a paradigm for food safety, with emphasis on risk reduction at each stage of food production from the farmer to the consumer. However, the high level of consensus about the appropriateness of this model has to be matched by meaningful analysis of how to achieve overall risk reduction[44]. We

will focus on PT *Salmonella* in this paper and the need for more research in the areas where the scientific literature is deficient. This will enable the development of effective control strategies to address those weaknesses and reduce human disease while enhancing poultry health and productivity.

2.2 Epidemiology of *Salmonella* in Poultry and Poultry Products

Salmonella can be found in virtually every part of the world and carried by an extremely wide variety of hosts including humans and other mammals, birds, reptiles, and insects[8,72,98,136]. Knowledge of the incidence and serotype distribution of salmonellae responsible for zoonotic disease transmission in domestic animal populations is essential for understanding the relationships within and between the reservoirs of salmonellae in animals and humans. Advances in poultry production practices changes in consumer lifestyles and preferences, and heightened nutritional awareness have all combined to make poultry products a leading source of protein for much of the world. Thus the incidence of *Salmonella* infection in poultry flocks and associated incidence of *Salmonella* contamination of poultry products are of considerable public health significance.

2.2.1 Prevalence of *Salmonella* in Poultry Flocks

Although salmonellae have been isolated in poultry flocks of various species, including both broiler and layer breeds, estimates of the prevalence of salmonellae in commercial poultry and their environments have varied considerably. A 1991 survey of poultry in the Netherlands reported that fecal samples from 94% of the meat-type broiler

flocks and 47% of the egg-type layer flocks in Netherlands were *Salmonella* positive[52,161]. Similarly, in 1994 the environments of 87% of turkey flocks in Canada were found to be *Salmonella* positive[86] and 53% of flocks tested from either fecal or eggbelt sampling in Canada were positive for *Salmonella* spp[130]. In the United States, studies of pooled cecal samples from spent egg-layers in the southern US, detected salmonellae in 100% of the flocks[168] and 86% of 406 layer houses from several regions[50].

It is difficult to compare the prevalence estimates obtained from different studies. While they may reveal true differences in the distribution of *Salmonella* across geographic regions and management systems, they may also be simply due to differences in the techniques used to determine the *Salmonella* prevalence. These data reveal that the apparent prevalence of *Salmonella* differs depending upon sample types, collection and handling methods and detection techniques. These differences may mask the impact of other factors such as raising practices, seasonal patterns and processing procedures that are actually causing true changes in the distribution of the bacteria. Aho, *et al.* reported that pooled samples were more effective to predict contamination than individual samples[3] and DeRezende, *et al* reported drag-swabs were more sensitive than individual litter samples[53]. Fecal samples are more sensitive indicators of *Salmonella* contamination than litter or other environmental samples where bacterial survival is affected by factors such as ambient temperature, airflow, available water (Aw) and pH[109]. Martinez-Urtaza, *et al* reported *Salmonella* contamination could vary on seasonal or temporal factors such as temperature, wind, hours of sunlight, rainfall and humidity in marine environments[111].

Strains of *Salmonella* responsible for poultry carcass contamination arrive at the farm when the chicks are placed. Bailey, et al reported that up to 98% of samples from

the hatchery were contaminated with *Salmonella* and in two trials found a significant association between serotypes found in the hatchery and those found on the final carcasses (7-36%).[11] *Salmonella* are ubiquitous and reducing the farm-level prevalence requires effective implementation and enforcement of management and biosecurity measures including proper poultry house design, adequate ventilation and reduced environmental humidity [53,109]. Production is the beginning of the food supply continuum and failure to control contamination at this stage of production increases the risk of foodborne illness for the consumer and the cost of risk reduction efforts at later stages of production and distribution.

2.2.2 Prevalence of *Salmonella* in Poultry Products at the Processing Plant

Salmonella contamination in poultry products at the processing plant is primarily due to cross contamination by physical contact during carcass processing such as improper cleaning and disinfection of processing lines, improper chilling and storage temperature, poor worker hygiene and infestation with rodents and insects.[103,149] *Salmonella* have been isolated from water, equipment, and carcasses in processing plants.[149] Lillard *et al* reported a significant increase in *Salmonella* incidence on carcasses exiting the immersion chiller when compared to other processing stages such as pre-scald (at bleed line), post-scald, post-pick and post-evisceration.[103] Venter et al reported that even in fully automated chicken egg layer management systems, bioaerosols can transmit *Salmonella* to eggs.[164]

PT salmonellae can be transmitted vertically to the progeny and horizontally within and between flocks[65,66,143] about 98% of hatchery samples were contaminated with

Salmonella.[10] Thus, *Salmonella* contamination in poultry products is a result of both infected birds entering the processing plant and contamination at the processing plant or during transportation[137]. Each stage in the production continuum has both direct and indirect effects on the *Salmonella* prevalence in finished poultry products. Carcasses from infected flocks can result in increased *Salmonella* prevalence but an effective HACCP plan at processing plants can reduce *Salmonella* loads. However, even with a lower *Salmonella* level in the flock, cross contamination during carcass processing with improper cleaning, disinfection, and chilling and storage temperatures can lead to high *Salmonella* loads in the poultry product. There is not enough data in the current literature to determine which stage of the production continuum is the most directly associated with human foodborne illness. But the literature does establish that the first step in the poultry product *Salmonella* contamination pathway is from infected flocks. This leads to cross contamination of transportation crates, carcasses, equipment, processing plant personnel, and vehicles and equipment used for retail-outlet processing, transportation and distribution.[130,137]

Transmission within the processing plant can however, be reduced by effective implementation of a hazard-analysis-critical-control-point (HACCP) program[152]. In 1996, the USDA, FSIS issued a mandate for implementation at processing plants and abattoirs throughout the United States. The national baseline pre-HACCP levels reported by FSIS were 20% for carcasses and 44.5% for ground meat in 1996.[153,155] After implementation of the HACCP guidelines FSIS reported a substantial decline in *Salmonella* prevalence for poultry and other meat products at processing plants[153,154,155,156]. According to FSIS reports, the prevalence declined to 11% for carcasses and 16% for ground chicken at US

processing plants in 2001.[157,159] As a result outbreaks traceable to errors in processing plants are rare and when outbreaks occur they are often associated with changes in processing or packaging technology whose effect is not determined before the product is on market.[63]

While the HACCP program has greatly reduced the level of contamination in poultry meat products at the processor, it has not been able to eradicate *Salmonella* from poultry meat products. Human factors are also important for cross contamination from production until consumption. Historically, most bacterial food poisoning in the US is associated with mishandling, either in the home or in the food service establishment. There is extensive information about what happens at processing, but there is little data on what happens post-processing from arrival at the retail distribution outlet up to the moment of consumption. After processing, the next stages in the production continuum for which data are available are reported incidences of illness and death post-consumption. Hence the period of time from processing through transport, distribution, further processing and retail sale is a "gap in the knowledge" related to the farm-to-fork production continuum. There are still opportunities for bacterial growth and cross-contamination of products during the process of transporting, storage, handling and retail distribution of poultry products.

There is a limited quantity of information in the literature regarding the prevalence of *Salmonella* in poultry products sampled from retail grocery store outlets, and there is also considerable variation in those reports. *Salmonella* have been isolated from 57% of chicken carcasses in Portugal,[105] 43% of ready-to-cook broiler carcasses from retail stores in Ohio,[18] and 29% of frozen broiler carcasses from retail stores in

Arkansas.[87] A recent study in the Washington DC area reports a *Salmonella* prevalence of only 4% from retail poultry carcasses.[177]

Just as the studies on *Salmonella* prevalence on poultry farms have been difficult to compare due to differing sampling and culture methods, these same issues have contributed to very different estimates of *Salmonella* prevalence in retail poultry products. The methods used for obtaining the samples from the poultry products were not consistent across the different studies. The potential impact of the differences in methodology has been documented in the literature. Uyttendaele and colleagues report a 100% increase in the *Salmonella*-positive samples when carcasses were cut into individual parts before sampling rather than using whole carcass-rinse samples.[160] Jorgensen and colleagues report that *Salmonella* was more frequently isolated from samples containing chicken skin in comparison with those containing carcass-rinse fluid only.[91] Differences between studies in the media used for enrichment, selective enrichment, and isolation can also affect estimates of prevalence. Estimating the prevalence of *Salmonella* in retail poultry products, identifying risk factors for elevated prevalence in different regions, and evaluating progress toward reducing the prevalence of *Salmonella* in retail products will require validated, standardized sampling and testing protocols.

2.2.3 Distribution of *Salmonella* Serotypes in Poultry Flocks, Products, and Human Salmonellosis

Although more than 2300 serotypes of *Salmonella* have been identified, only about 10% of these serotypes have been isolated from poultry.[72] Moreover, an even

smaller subset of serotypes accounts for the vast majority of poultry *Salmonella* isolates. The distribution of *Salmonella* serotypes from poultry sources varies geographically and changes over time. Several serotypes are consistently found at a higher incidence.

According to the report from the U. S. Department of Agriculture (USDA) National Veterinary Service Laboratory, the most commonly identified species in chickens in the United States were *S. heidelberg, S. enteritidis, S. hadar, S. montevideo, S. kentucky and S. typhimurium.*[58,59,60] The significance of poultry as a reservoir for human salmonellosis can be illustrated by considering the species commonly isolated from humans. The most often reported to the Centers for Diseases Control and Prevention (CDC) from human sources in the United States were *S. typhimurium, S. enteritidis, S. heidelberg, S. hadar, S. newport, and S. agona.*[14] The specific prevalence of *S. enteritidis* has been a topic of considerable interest in recent years due to the epidemiologic association of salmonellosis and consumption of contaminated eggs. The increasing public health significance of *S. enteritidis* was shown in a survey of the frequency of reporting of human infections with various *Salmonella* in 21 nations and 10% of theses nations reported *S. enteritidis* as their most common species in 1979 to 43% in 1987.[133] Murase, *et al.* reported *S.enteritidis, S.cerro, S.montevideo* and *S. mbandaka* were isolated from about 60 % of layer house environmental positive samples.[121] *Salmonella* isolates obtained from the drain water collected after the washing of the eggs in the egg-processing facility were the same serotypes found in the chicken houses. Knowledge of *Salmonella* serotypes is important to identify sources and routes of contamination not only for control and prevention but also for outbreak investigation and vaccine development.[145]

2.2.4 Risk Factors for Transmission of *Salmonella* in Poultry

The physiology of Salmonellae has contributed to difficulty in controlling environmental contamination and transmission of the organism. Salmonellae are hardy bacteria with several potential vehicles, vectors, and reservoirs within a poultry flock. They are facultative anaerobes and can grow well under both aerobic and anaerobic conditions.[72]The optimum temperature to support growth is 37°C, but they can grow over a range of 5 to 45°C and can grow within a pH range of 4.0 to 9.0, with an optimum pH 7.0.[72]

Hardiness

The environmental persistence of PT salmonellae is a significant factor in the epidemiology of *Salmonella* in poultry by creating opportunities for horizontal transmission of infection within and between flocks. *S. heidelberg* can be isolated from contaminated litter after 7 months of holding at room temperature.[141] Williams and Benson *et. al.* reported *S. typhimurium* can survive for 16 months in feed and 18 months in litter stored at 25°C.[174] Salmonellae can be introduced into poultry flocks from many different sources. The extremely wide host range of PT salmonellae create an equally large number of reservoirs of infectious organisms from which PT salmonellae can be transmitted to chickens or turkeys.

Insect and Animal Vectors for *Salmonella*

Kopanic and McAllister *et al.* reported cockroaches and lesser mealworms could carry *Salmonella* internally and externally and spread them throughout the poultry

house.[96,112] Non-biting flies, fleas, ticks and bread beetles were also reported to be *Salmonella* vectors in feed and feed mixtures.[16,25,38] Mice have been also important vectors for *S. enteritidis* in laying hens.[78] Wild birds and lizards were recorded as reservoirs of *Salmonella* and can directly and indirectly cause transmission to poultry flocks.[35,138] Insect and animal vectors are widely believed to be sources of bacterial contamination in feed, poultry products and even directly to human salmonellosis.

Feed as a Source of *Salmonella*

Contaminated feed, perhaps through insect and animal vectors or as a direct source of bacteria itself, is also often implicated as a source of *Salmonella* in poultry flocks[55,85]. Four of eight *Salmonella* serotypes isolated from a turkey breeding facility over a 5-yr period had also been isolated from samples of pelleted feed.[176] In Australia, *Salmonella* serotypes, which were not previously detected in flocks, were detected first from raw feed ingredients. These serotypes then later appeared in live birds and processed carcasses.[106] Cox et al. reported Salmonellae isolates in 92% of meat and bone meal samples and in 58% of finished feed (mash) samples from commercial mills in the United States.[33]

Salmonella contamination is a widespread problem in the feed industry. Since September, 1990, the Food and Drug Administration – Center for Veterinary Medicine (FDA-CVM) announced a goal of *Salmonella*-free animal feed ingredients and finished feed. CVM conducted feed surveys of animal feeds and protein ingredients at feed mills and on-farm mixers in 1993 and 1994.[113] A total of 151 feed meal samples were collected and analyzed for *Salmonella* contamination, in which 56 % (57/101) from animal protein

and 36% (18/50) from vegetable protein samples were positive for *Salmonella* in the 1993 survey. As a result of the findings from this survey, CVM recommended application of a HACCP program to feed ingredients and feed manufacturing. In addition, FDA approved two food additives, radiation, and formaldehyde, for the specific purpose of controlling *Salmonella* in feed. The CVM survey data indicated that transmission of *Salmonella* from meal to feed is very likely, that transmission is reflective of the prevalence in protein meal and that processing or addition of medication can reduce the contamination.[113]

In the 1994 survey, CVM collected 187 samples of which 93 were feed and 94 were protein meal from feed mills and on-farm mixers. The prevalence of *Salmonella* in protein meal samples (48.4%) was about twice that of feed samples (24.7%)[113]. This was consistent with previous survey results and that pattern of increased bacterial contamination in animal-protein feed ingredients was also reported in more recent surveys. In 2002 and 2003, FDA conducted a feed commodity survey of 122 rendered animal by-product samples, and 79 plant-based protein product samples. Overall, animal by-product samples had contamination prevalence of 84% for *Enterococcus*, 34% for *Salmonella* and 40% for *E.coli*.[165] Plant –based protein products had contamination prevalences of 91%, 5%, and 43% for *Enterococcus*, *Salmonella*, and *E.coli* respectively. These studies also concluded that *Salmonella* contamination was more prevalent at commercial feed mills than at on-farm mixers. This was perhaps because on farm mixers were small in volume compared to commercial feed mills, samples collected at commercial mills contained a greater percentage of animal-based protein than the on-farm mixer samples, and meal was more likely to become contaminated at commercial

feed mills than at on-farm mixers. It was not surprising that both studies demonstrated that *Salmonella* contamination of plant-based protein was less than animal-based protein. However, the level of contamination in plant protein feed samples was much higher than expected. While the prevalence of *Salmonella* contamination in plant-based feed is lower than that in animal-protein feed products, the magnitude of the impact of plant-based feed on the distribution of *Salmonella* in livestock is greater due to the greater exposure that livestock have to plant-based feed. In the FDA surveys, only 37% of the feed contained animal protein at the commercial feed mill and 12 % of the feed from the on-farm-mixer samples contained animal protein. Overall in the US, 65 % of feed in feed mills and about 90% of feed in on-farm mixers are plant-based proteins. Thus, plant and vegetable meal are the main ingredients for animal feed. Among the plant-based protein, soybean, sunflower and cottonseed meals had a higher prevalence of *Salmonella* contamination than other meals. These data demonstrate that animal feed and meal are still contaminated with *Salmonella* and other enteric bacteria. Further research is necessary to achieve the FDA goal of *Salmonella*-free feed and meal.

There are several potential mechanisms for plant-based feed to be a source of *Salmonella* contamination to poultry. The obvious route of contamination may of course be that feed attracts animal and insect vectors that are *Salmonella* carriers. As previously discussed several species of rodents, insects, reptiles, and wild birds may serve as vehicles for *Salmonella* either directly or indirectly by contaminating the poultry feed.

Another theory is that application of litter, manure, municipal sludge and other agents may be seeding the fields with enteric bacteria including *Salmonella*. Then either the surface of the plant is contaminated or the bacteria are actually taken up into the

vascular system of the plant. Ayanwale *et al* reported corn silage which was grown on land fertilized with sewage sludge was contaminated with *Salmonella*.[9] Bean and pulp are often contaminated with *Salmonella* and even different sanitization methods were shown to be highly variable as an effective means of reducing *Salmonella* counts.[119] Jones *et al* reported feed ingredients and dust were major sources of *Salmonella* contamination at commercial feed mills.[90] Penteado and Otirroaga *et al* reported *Salmonella* could be internalized into fresh mangos during post harvest insect disinfection procedures and *Salmonella montevideo* can attach to the surface of tomatoes within 90 minutes.[126,128] Hundreds of millions of tons of plant-based feed are used in the poultry industry. Determining the best methods to handle, store and treat plant-based feed to eliminate these pathogens is important for the feed and livestock industries.

Chickens as a Reservoir for *Salmonella*

PT salmonellae can be transmitted vertically to the progeny of infected breeder flocks and horizontally within and between flocks.[65,66,143] Vertical transmission of PT salmonellae to the progeny of infected breeder flocks can result from the production of eggs contaminated by salmonellae in the contents or on the surface.[73] During oviposition, eggshells are often contaminated with PT salmonellae by fecal contamination. The penetration of salmonellae into or through the shell and shell membranes can result in direct transmission of infection to the developing embryo or can lead to exposure of the chick to infectious *Salmonella* organisms when the shell structure is disrupted during hatching.[72] Some PT serotypes, particularly *S. enteritidis*, can be deposited in the contents of eggs before oviposition. Transovarian transmission of *Salmonella* to progeny is an

important aspect the epidemiology of *S. enteritidis* in chickens. Also, any PT salmonellae carried in or on eggs can be spread extensively in the hatchery.[10] As chicks pip through eggshells, salmonellae are released into the air and circulated around hatching cabinets on contaminated fluff and other hatching debris. Cox et al. reported that 12 different serotypes of salmonellae were isolated from more than 75% of egg fragments, belting material, and paper pad samples from three broiler hatcheries.[30]

Horizontal transmission can occur by direct bird-to-bird contact, ingestion of contaminated feces or litter, contaminated water, personnel, farm and personal equipment, and a variety of other sources.[73,100,122,123,176] Snoeyenbos *et al.* reported, horizontal transmission could occur when unexposed day-old chicks were raised together with infected day-old chicks.[142,143] Also, reported by Gast and Beard *et al., S enteritidis* could be found in the feces and internal organs of uninoculated laying hens housed in cages adjacent to those of orally inoculated birds.[65,66,67] Contaminated poultry house environments are identified as one of the major implication of PT salmonellae[97]. Since the chicken itself is a reservoir of *Salmonella*, control measures need to start from the hatchery. Bailey *et al.* reported about 98% of samples from the hatchery were contaminated with *Salmonella* in two trials.[10] Early mortality rates for infected chicks were higher and the survivors continue to carry *Salmonella* and shed the organism intermittently. Therefore, a successful intervention program for *Salmonella* must be multifaceted, with one component being disinfection in the hatchery. One attempt by the industry to develop a comprehensive approach to *Salmonella* control is the National Poultry Improvement Plan (NPIP).[125]

The NPIP was developed in response to early efforts to improve the economic aspects of poultry production through breeding and disease control. Initially started to control pullorum disease and fowl typhoid, which are caused by *S. pullorum* and *S. gallinarum* respectively, by selection and testing of chickens and turkeys,[124] it was later expanded to include *Mycoplasma synoviae*, Avian influenza, *S. enteritidis* and other *Salmonella spp.*[125] However, PT salmonellae are ubiquitous and can be harbored by a wide variety of hosts who can transmit the bacteria to poultry. NPIP has had limited success in its ability to reduce PT salmonellae contamination of poultry.

2.3 Detection

Salmonella prevention is important for consumer health, poultry health and the food industry. Rapid and reliable monitoring and screening programs can help reduce the incidence of *Salmonella* infection in poultry and humans that consume poultry products. However, these programs require adequate *Salmonella* detection tools. Detection of *Salmonella* plays several important roles:

1) Determining the scope of the problem at each level in the food supply continuum,
2) Quantifying the risk to human health at each level,
3) Quantifying the economic costs to the industry,
4) Evaluating control measures,
5) Monitoring and surveillance, and
6) Early alert system to recall product prior to consumer illness.

Conventional bacterial culture methods are still used most often to identify *Salmonella* and require at least 3-11 days.[146,147,166,167,169] These methods are time

consuming and labor intensive and may have low sensitivity for the identification of samples with low initial numbers of *Salmonella*, as may often be seen in sub-clinically infected chickens, resulting in false-negative test results.[64]

Salmonella can be detected from a variety of sources including: poultry tissues, eggs, litter, and ready-to-eat products. PT salmonellae are highly invasive and can be systemically disseminated within the infected bird to numerous internal tissues, including the liver, spleen, ovary, oviduct, testes, yolk sac, heart, heart blood, kidney, gall bladder pancreas, synovia, and the eye[72]. Each of these sites can provide samples for diagnostic culturing. *S. entertidis*, can be deposited in the contents of eggs before oviposition.[65] Culturing eggs for *S. enteritidis*, has been used as a test for assessing the potential threat to public health posed by infected laying flocks[51,52]. Gast *et al.* reported that culturing pools of egg contents detected experimentally infected hens at a frequency similar to culturing fecal samples or testing for specific serum antibodies during the first two weeks after inoculation.[68] In a survey of birds submitted to a diagnostic laboratory, 78% of the chickens and 70% of the turkey intestine samples were found salmonella positive.[23,54,86] In experimentally inoculated laying hens, *S. enteritidis* was recovered more often from the intestinal tract than from any other tissue sampled.[69]

Fecal shedding of salmonellae into the poultry house environment by infected birds makes culturing environmental samples a useful diagnostic tool. Moreover, environmental samples also provide an opportunity to monitor the introduction of salmonellae into poultry houses by vectors, personnel, equipment, and other sources. Fresh feces are the most sensitive sample to detect the shedding of salmonellae.[80] Testing for litter samples can sometimes provide a comparable level of detection.[135] Kingston and

De Rezende *et al.* reported drag-swab samples were more sensitive than litter samples in detecting salmonellae.[53,95] Other environmental samples such as cage surfaces, water sources, eggbelts, trapped rodents, and even dust were useful to detect salmonellae contamination. Dust can remain contaminated with salmonellae even after cleaning and disinfection of poultry houses.[79,80] Hatchery fluff is frequently contaminated with salmonellae, and useful for early detection of infection in flocks.[118,135] Culturing poultry feed for salmonellae are often important in establishing the source of infection of a flock with a particular serotype.[142] Tissue samples, especially neck skin and ground meat samples were taken from the processing plant and samples from ready-to-eat products were checked for *Salmonella* contamination[91].

Generally, most of the conventional culture methods involve four principal stages for isolation and identification of PT salmonellae: pre-enrichment, selective enrichment, selective plating, and confirmation. First, nonselective pre-enrichment is used to encourage the growth of very small numbers of salmonellae or to allow the recovery of injured *Salmonella* cells. Pre-enrichment is not favorable when testing samples with large numbers of competing organisms such as those found in intestinal contents or feces that might overgrow salmonellae. Second, selective enrichment is used to allow additional expansion of the *Salmonella* population while suppressing the growth of other organisms. Third, plating on selective agar media is used to obtain isolated colonies, each derived from a single cell. Fourth, colonies with appearances characteristic of salmonellae are subjected to biochemical and serologic tests to confirm their genus and serotype identity.

Generally, samples from all stages of the production continuum require plating on selective media and confirmation of the isolates. The enrichment requirements vary

according to nature of samples. With the exception of intestinal tissue or contents, tissue samples from infected birds contain relatively low numbers of competing organisms, thus they are often transferred directly to either selective or non-selective agar media. Eggshells are generally sampled without pre-enrichment. Whole eggs can be sampled by aseptic breaking to release the contents and later manual crushing and addition of selective enrichment broth.[70,71] Environmental samples such as litter and dust are generally collected in sterile plastic bags, and other environmental samples, such as various surfaces can be sampled using moistened gauze pads. Animal feed can be tested by collecting several representative samples from each lot and transferring into selective enrichment broth. Pre-enrichment of poultry feed samples has been reported to be unnecessary or even counterproductive.[31,32,40]

Humphre and Whitehead *et al.* reported pre-enrichment with nonselective media to be useful for recovering small numbers of *S. enteritidis* from egg contents[83] and shorter selective enrichment (6-hrs) has been used successfully to recover salmonellae from animal feed.[40,41] Nonselective culture media include buffered peptone water (BPW) and trypticase soy broth (TSB) are generally incubated at 37°C for 24 hrs and later transferred to selective enrichment cultures such as Tetrathionate Hanya (TT- H) or Rappaport Vassaliadis broth (RV) and incubated at higher temperature (41-43°C) to suppress the growth of competing microflora for 24hrs.[41,42,107] Waltman, *et al.* reported delayed secondary enrichment, in which selective enrichment broth cultures are held for an additional 5 days at room temperature to allow salmonellae to grow to detectable levels, to improve the recovery of PT salmonellae from poultry diagnostic and environmental samples.[167,169] Selective media are transferred to agar media and incubated at 37°C for

24-48 hrs. Commonly used plating media are brilliant green (BG) agar, XLD agar, XLT4 agar, and bismuth sulfite agar. Presumptively positive colonies from selective agar plates are tested to confirm genus identity and to determine serotype. Triple sugar iron agar (TSI) and Lysine-iron agar (LIA) were used to identify PT salmonellae. The serotype can then be determined by slide agglutination tests with monovalent antisera to specific "O" antigens and tube agglutination tests with antisera to flagellar "H" antigens[72].

The standard culture method requires at least 3- 5 days to isolate and identify salmonellae. Other comparatively more rapid techniques have been proposed and investigated in recent years, but to date none of these tests are standardized or widely accepted[102]. Many detection techniques and selective media were developed to detect *Salmonella* rapidly and accurately in recent years but still need to be improved before they can be proven to be reliable, simple and cost effective enough to perform in field conditions. Specific antibodies to salmonella antigens have been used to develop a variety of enzyme-linked immunosorbent assay (ELISA) methods using monoclonal and polyclonal antibodies to *Salmonella* lipid polysaccharide (LPS) or flagella[76,93,94] but these methods are reportedly not quite as sensitive as conventional culture.[147]

One application of antibodies involves coating small magnetic beads with specific antibodies, which are then used in an immunomagnetic separation method[37,61]. The antibody-coated beads bind to any *Salmonella* target antigens present and a magnetic field can then be applied to recover the bead-antibody-antigen complex.[37] Another approach to rapid testing for salmonellae in recent years, DNA hybridization, involves using particular DNA sequences unique to salmonellae. Hybridization of a probe with DNA extracted from the sample indicates a positive result.[77]

The development of polymerase chain reaction (PCR) technology and real time PCR (RTPCR) have allowed the specific amplification of particular target segments of DNA.[26,27] Recently, a lot of attention has been focused on PCR techniques to detect *Salmonella*. While promising results have been reported in the literature, these tests still need to be validated and the sensitivity and specificity need to be demonstrated on field samples. This will also require that uniform protocols for sampling, handling, and processing be developed. Gouws *et al.* reported PCR test used with *Salmonella* specific ST 11 and ST 15 primers and targeted for 429 base pair (bp) region of DNA and PCR tests were quite sensitive and specific in artificially challenged samples, however the results for naturally contaminated samples were not reported.[74] In Europe, PCR detection of *Salmonella* contamination in raw poultry was tested with PCR specific primer pairs (Sigma-Genosys, Cambridge, UK) used to amplify fragments within 1.8 kb *Hin*dIII DNA sequence specific to a wide range of *Salmonella* serotypes.[171] In both studies, the sensitivity and specificity of PCR were not calculated and only reported as highly sensitive compared to culture and having a high rate of concordance with culture. Several researchers have used different *Salmonella* specific DNA probes such as an 16S rDNA and iro B specific DNA probe for PCR testing.[13,104] Amplification of nucleic acids by PCR has become a powerful diagnostic tool for microbial infection and microbial contamination in food samples. However, lack of validation and standard protocols, as well as variable quality of reagents and equipment, limit the efficient application of PCR from research laboratories to end user field laboratories. In 1999, the European Commission approved the research project, FOOD-PCR (http://www.PCR.dk), which aims to validate harmonization procedures and standardization criteria for detection of

foodborne pathogens by PCR further indicating the need to validate and standardize PCR methods.[13]

2.4 Control and Prevention

PT salmonellae can be effectively controlled by coordinated and simultaneous interventions on the problem from several directions. At the farm level, eggs and chicks or poults should only be obtained from *Salmonella*-free breeding flocks. Hatching eggs should be properly disinfected and hatched according to stringent sanitation standards.[66,68,69,71] Poultry houses should be thoroughly cleaned and disinfected. Rodent and insect control measures should be incorporated into house design and management and verified by periodic testing.[124,154] Rigidly enforced biosecurity practices should be implemented, restricting entry onto poultry housing premises to only authorized personnel and equipment, preventing horizontal transmission of salmonellae between houses.[116,150] Only pelleted food or feed containing no animal protein should be used, to minimize contamination.[174,175] Treatments such as medication, competitive exclusion cultures, or vaccination can be applied to reduce *Salmonella* susceptibility.[28,72,125] Frequent testing of poultry and environmental samples has also reportedly been successful for *Salmonella* control in the poultry industry. Such coordinated control programs have reportedly been successful in addressing *Salmonella* problems in both chickens and turkeys.[52,114,129]

At the processing stage, implementation of a Hazard Analysis and Critical Control Points (HACCP) Plan has been effective in reducing *Salmonella* contamination of carcasses[133]. The United States Department of Agriculture (USDA), Food Safety

Inspection Services (FSIS) conducts annual surveillance of microbial contamination in processing plants and slaughterhouses to monitor microbial contamination of carcasses. Since implementation of the HACCP program, they have been able to demonstrate that the *Salmonella* prevalence on carcasses in the processing plant was significantly lower than before implementation.[134] However, state or county departments of health loosely regulate transportation and retail distribution of processed chicken products. Surveillance usually only occurs in response to outbreaks. Effective development and implementation of HACCP and surveillance programs to monitor microbial contaminations at the retail store level are needed to close that final gap in the *Salmonella* control spectrum before the product reaches the consumer.

Other measures, such as gas treated packaging, irradiation, organic acid treatment, and biofilm treatments have proved to effectively lower or inhibit salmonella and other microbial growth in products. Potential pre-harvest interventions to control *Salmonella*, or other foodborne hazards, need to be considered in terms of cost, impact and probability of post intervention contamination. The technical feasibility of pre-harvest control of *Salmonella* using microbiological testing and regulation has been demonstrated by the Swedish poultry and swine industries[44]. However, perhaps the most eloquent statement of the difficulty and cost of implementing the 'Swedish model' for *Salmonella* control is that, despite its apparent success, after 40 years it has not been adopted by any major swine or poultry producing nations because improvement of any single factor only had a limited impact on the level of contamination, and the largest reduction was observed when several factors were improved concurrently.[45] As one would expect, lack of data was identified as a major limitation to the development of effective control models.

2.5 Discussion

Generally, the food supply continuum can be divided into four sectors: production, processing, distribution and consumption. Within each sector, a range of interventions might be applied to reduce risk of foodborne hazards, and the goal is to define the optimal mix of interventions across the continuum that delivers maximum risk reduction at minimal cost. Ideally, interventions should be applied strategically at those points where the greatest impact on ultimate risk will be achieved. However, participants in all sectors should be motivated by their own self-interest to ensure that they take what measures they can to reduce risk. Public education should reinforce that the food supply will never be risk free, and that appropriate kitchen hygiene and cooking practices are powerful tools for mitigating risk of multiple biological hazards. Processors want to be meticulous in development and implementation of good management practices and process control procedures, as brand image can be destroyed almost overnight by adverse events, such as disease outbreaks or product recalls. Animal producers are ethically bound to reduce foodborne hazards in animals under their care. Also processing companies must meet regulatory goals for microbiological safety, but for commercial reasons they may need to meet more stringent national or international standards.

Raw poultry products are contaminated with harmful, pathogenic and spoilage bacteria from farm to table by infected stocks, cross contamination, improper handling, storage or cooking of poultry, which can lead to human foodborne illness and loss of product shelf life[140]. Food safety regulations on meat and poultry are important issues in an industry where production costs must be minimized to maintain economic viability. However, efforts to enhance product quality and comply with food safety regulations

often result in increases in production costs. In 1996, FSIS updated the final rule on pathogen reduction and HACCP systems. The new regulatory measures address hazards within slaughter and processing plants, but these measures must be part of a comprehensive food safety strategy that addresses hazards at all levels in the farm-to-fork model. These levels include the improvement of food safety at the animal production and intermediate stages before the slaughter plant, food safety during transportation, storage and retail sale, and educating consumers to follow safe food handling practices such as proper storage, preparation, and cooking of meat and poultry products. Each of these components of a comprehensive *Salmonella* control strategy is essential to achieving the goal of minimizing the risk of foodborne illness.

There is a lot of information about the epidemiology of *Salmonella*. Control strategies have been developed that effectively reduce *Salmonella* prevalence in processing plants or on individual farms. However, human *Salmonella* incidence is not decreasing and there are still gaps in the available information from the literature. Focusing on the pre-consumer aspects of the poultry production continuum, this literature review has identified 4 areas that need additional research before we can expect to achieve a reduction in the incidence of human foodborne salmonellosis in the US:

1) About 80% of feed is plant-based. What role does plant-based feed and field management play in the transmission of *Salmonella* by surveillance of animal feed study?

2) The chicken house is a reservoir for *Salmonella*. How can we best manage that environment to inhibit *Salmonella* growth and transmission?

3) What is the most sensitive and specific standardized protocol available to rapidly detect natural *Salmonella* contamination of retail chicken meat?

4) There is a lack of information about prevalence of *Salmonella* contamination of poultry products once it leaves the processing plant. Since this is the final step before it reaches the consumer, it may indeed be the most important because failure here will result in direct exposure to the public and will un-do any progress made before. It is also an excellent opportunity to enhance previous control efforts by making a final reduction in contamination before the product goes to the consumer. What is the prevalence of *Salmonella* contamination of poultry products at the retail store and what are the risk factors for contamination?

In conclusion, a lot of research has been conducted on various aspects of the epidemiology of *Salmonella* in poultry products and its role in human foodborne salmonellosis. However, the problem is still there and may be growing, therefore more research is needed. Failing to act now will only increase the costs of foodborne illness, both economically and in terms of human suffering. As the US population ages and more people are likely to be immunocompromised due to advanced age, immunosuppressive disease or medical treatments that result in immunosuppression, the importance of enhancing the microbial safety of the nation's food supply will only increase.

3. Chapter II: Estimating the Prevalence of Enteric Bacterial Contamination of Plant-Derived Animal Feed and Milk Replacer in Maryland and Northern Virginia

3.1 Abstract

Animal feed is at the beginning of the food safety chain in the "farm-to-fork" model. Contaminated animal feeds have been identified as high-risk vehicles for the introduction of infectious agents of public health significance to commercial poultry flocks. A cross-sectional study was undertaken to estimate the prevalence of enteric bacteria*, Enterococcus*, *E.coli, Salmonella*, and *Campylobacter* from 158 plant-derived animal feed and milk replacer samples. Feed was collected from 8 retail animal feed stores located in northern Virginia and the state of Maryland from January- 2002 to September- 2002. All samples were negative for *Campylobacter*. One sample (0.6%) was positive for *Salmonella* and nine samples (5.7 %) were positive for *E.coli*. Eighty samples (50.6%) were positive for *Enterococcus*. Samples collected in winter (January-2002) were negative for both *E.coli* and *Salmonella*. One sample was positive for *Salmonella*, and nine samples were positive for *E.coli* from the feed samples collected in summer (August and September-2002). Positive samples included: whole corn, cracked peanut, cotton seed, mixed grain horse feed, and milk replacer. The *E.coli* and *Enterococcus* prevalence in animal feed purchased between the months of August and September of 2002 had a 38-fold increase in the risk of contamination by *Enterococcus* than those samples purchased between the months of January and February 2002 ($p<0.001$). Risk factors associated with the environmental conditions that the feed

samples were exposed to during harvesting; transportation and storage may contribute to what appears to be a seasonal pattern of contamination. While the study design and sample size did not permit a complete analysis of seasonal trends, these data are consistent with other studies, indicating that assessing the prevalence of bacterial contamination of feed will require a study design that takes sampling season and day of the week into consideration when developing the sampling framework. Failure to do so may result in biased estimates of prevalence and associated risk factors.

3.2 Introduction

Animal feed and animal feed ingredients have a substantial and far-reaching impact upon global trade. According to United Nations Food and Agriculture Organization records, more than 100 countries imported a total of 2 million tons of meat and meat meal alone in 1999.[62] Animal feed is a potential vehicle for introducing microorganisms to livestock. Animal feeds and especially their animal by-product components are known to be primary sources of microbes to food animals such as cattle, poultry, and swine. Feed and water are the primary sources of bacterial pathogens in food producing animals.[115] Thus, animal feed is an important early link in the "farm-to-fork model" of food safety[158]. The emergence of variant Creutzfeldt-Jakob disease has raised awareness of the importance of contaminated animal feed, but less attention has been paid to the role of bacterial contamination of animal feed in human foodborne illness.[20,36] Bacterial contamination of animal feed can lead to infection and colonization of livestock and poultry with these pathogens that may then be transmitted through the food chain to humans potentially causing human foodborne illness. Williams and colleagues concluded from their review of the incidence, distribution, and sources of *Salmonella* in poultry feed that there seems to have been little change in the *Salmonella* status of ingredients and poultry feeds over the last 40 years.[175] This paper provides ample evidence that *Salmonella* contamination has been and continues to be a problem in poultry feed.[175] Allred *et al*. surveyed 13,000 samples from 700 mills to determine the prevalence of *Salmonella* in feed and feed ingredients, 5% of feeds sampled were positive for *Salmonella.*[4] However, there is currently no consensus in the literature regarding the reported prevalence of *Salmonella* in finished poultry feed and feed ingredients.

Estimates have varied widely between studies with prevalence ranging from 0% to 78%.[12,89,161,] It is difficult to compare these differences in *Salmonella* prevalence estimates because the studies were conducted in several different geographic areas including Thailand, the Netherlands, and the United States. In addition, these studies each employed different bacteriological protocols and sampling methods. Feed microbial contamination has become an important issue due to concern over bacterial contamination of animal products.[4,163,172] Mitchell *et al.* from the FDA, Center of Veterinary Medicine reported that the tolerance for *Salmonella* in animal feed is inappropriate and it makes little difference whether the prevalence in production lot of feed is 5% or 95%.[117] *Salmonella* contamination is a widespread problem in the feed industry and the control and elimination of *Salmonella* during milling procedures has proven to be difficult.[172] In 1990, the US FDA Center for Veterinary Medicine (CVM) announced a goal of *Salmonella*-free animal feed ingredients and finished feed.

The CVM has reported results of surveys of microbial contamination in animal feed from both feed mills and on-farm mixers. Based on these findings, they have issued regulations to control contamination of animal feed. Since 1993, the CVM has conducted three surveys of processors that manufacture animal and vegetable sourced protein products used in animal feeds to determine the prevalence of *Salmonella* in these products. In the 1993 survey, 151 animal feed samples from 100 animal and 68 vegetable protein processors were collected. The *Salmonella* prevalence in animal protein was 56% compared to 36% in vegetable protein.[113] The 1994 survey found that 24.7% of feed samples and 48.4% of protein meal samples were contaminated with *Salmonella.*[113]

In the 2003 survey, 122 animal and 79 vegetable protein samples were collected and tested for *Enterococcus, Salmonella* and *E.coli.* The prevalence in animal protein was 84%, 34% and 40% respectively. In vegetable protein they were 91%, 5%, and 43% respectively.[163] The estimated level of enteric bacterial contamination from the animal sourced protein was not surprising since these organisms are routinely isolated from the gastro-intestinal tracts of livestock and poultry. The surprising result was the higher than anticipated prevalence from plant protein feed sources. The FDA-CVM survey findings were also not consistent with the other rather limited data reported in the literature. Crane *et al*. conducted a study to determine the *Salmonella* prevalence in commercial feed mills in the US and found that only 2% of incoming ingredients were contaminated with *Salmonella* and without meat meal, only 0.7% (2/300) were positive.[34] Despite the inconsistencies in the literature and the importance of vegetable protein as a main ingredient in animal feed, there are very few studies in the literature that focus on the prevalence of and risk factors for microbial contamination of vegetable protein in animal feed.

In the United Sates, the animal feed industry produced about 120 million tons of animal feed in 2000. Farmers spent approximately $ 25 billion on animal feed and almost 80% were plant-based products.[56] Known variables such as environmental conditions including temperature and relative humidity[111] affect the ability of microbes to survive in animal feed[131,132]. Further research is needed to identify risk factors for contamination, which may include crop production and storage practices. One common practice that needs further scrutiny is that of growing feed on fields in which unprocessed livestock manure or poultry litter have been applied.

A HACCP analysis of risk factors for contamination of livestock and poultry feed can be modeled after the human food safety farm-to-fork paradigm. In this case the "field-to-trough" model of feed production has several pathways in which enteric bacterial contamination of plant-sourced animal feed can occur. Prior to processing potential sources of bacterial contamination include: crops being splashed or irrigated with contaminated water and use of contaminated harvesting, hauling, and transport vehicles.[55,85,132] Cross contamination can occur during the extraction and milling processes; if target temperatures are not uniformly achieved by the feed during processing or if there is poor hygiene in the processing plant.[17,132] Even if adequate processing and hygiene procedures are adhered to, post-processing contamination during packaging and storage especially by insects, vermin or wildlife will also result in a product that may be contaminated by enteric pathogens.[31,33,42] Once delivery to the farm has occurred, the risk of contamination may be increased due to the ubiquitous presence of insects, vermin, and wildlife in association with bulk feed storage facilities and feeders.

Investigations of food-borne disease outbreaks traced back to fruit, vegetables, or grain have usually presumed that the surface of fruit, vegetable, or grain was contaminated with bacteria perhaps through water sources contaminated with manure or fecal matter, or through contact by contaminated agricultural personnel.[9] However, recent reports in the literature have indicated that leafy vegetables may actually take up the bacteria into the internal structures of the leaf and accumulate them in the plant.[120] If this does indeed occur, surface disinfection of fruit, vegetables, and grains will not be an effective means of ridding these products of bacterial contaminants.

This project is one of the very few studies conducted to assess the prevalence of microbial contamination in plant-based feed products at the level of the retail feed outlet. *Enterococcus* and non-pathogenic *E.coli* were included in this study because these bacteria are less fastidious than other pathogens such as *Salmonella* and *Campylobacter* and can be indicative of fecal contamination of feed. Collecting feed samples from retail animal feed store allows determination of the prevalence of these enteric bacteria in feed post-processing at a large scale feed mill, after packaging and shipping and yet prior to distribution to a farm in which on-farm management has a great influence on the observed level of contamination.

Objectives:

The objectives of this study were:

- To estimate the prevalence of microbes (*E.coli, Salmonella, Campylobacter* and *Enterococcus spp.*) in vegetable protein for animal feed and milk replacer samples purchased during winter and summer of a single year.

Hypotheses:

- The prevalence of *E.coli, Salmonella, Campylobacter* and *Enterococcus spp.* contamination in retail plant-based animal feed is greater than or equal to 10% prevalence.
- There is no significant difference in the prevalence of *E.coli, Salmonella, Campylobacter* and *Enterococcus spp.* contamination in retail plant-based animal feed whether purchased in winter or in summer.

3.3 Materials and Methods:

A cross-sectional study was conducted to estimate the prevalence of microbes (*E.coli, Salmonella, Campylobacter* and *Enterococcus*) and antibiotic resistance of enteric pathogens in plant derived ingredients and milk replacer for animal feed from 8 retail animal feed stores located in northern Virginia and Maryland.

Sample collection: Two sample collection periods were established. During the first collection period 80 samples were purchased in winter from January through February 2002. The second sample collection period consisted of 78 samples purchased in the summer, from August through September 2002. A total of 158 vegetable protein samples including: corn (whole, cracked, coarse), sunflower seeds, safflower seed, soybean meal, cotton seed meal, cracked peanuts, rice bran, mixed grain horse feed and milk replacer samples were collected from 8 retail animal feed stores located in the states of Maryland and northern Virginia. From each sample, 2 sub-samples of 100 grams each were collected using aseptic techniques in accordance with the Investigations Operational Manual (IOM) standards.[5] Each sub-sample was analyzed as stated in the 8th edition of the Bacterial Analytical Manual (BAM) for *E.coli, Salmonella, Campylobacter*[6,81,84] and *Enterococcus* species.[75]

Data Analysis:

Data were stored in Microsoft Excel spreadsheets and calculated for descriptive analysis. Analysis of Variance, Fisher's exact and chi-square tests[39]were conducted using analytical software – (Statistix-8, Tallahassee, FL). Sample sizes were calculated using

Epi-info 2000 software from CDC. The sample size was calculated to detect a risk ratio of 3 or greater.[47]

3.4 Results:

All samples were negative for *Campylobacter*. One sample (0.6%) was positive for *Salmonella* and nine samples (5.7 %) were positive for *E.coli*. Eighty samples (50.6%) were positive for *Enterococcus*. All feed samples purchased in the winter were negative for both *E.coli* and *Salmonella*. Feed samples purchased in the summer had one sample (sunflower meal) that was positive for *Salmonella*, and nine samples that were positive for *E.coli*. Results for microbial contamination are detailed in Table 1. Positive samples from the summer batch included: whole corn, cracked peanut, cotton seed, mixed grain horse feed, and milk replacer. Most samples collected during the summer were positive for *Enterococcus* (Table 2). The animal feed purchased during the summer had significantly greater prevalences of *E.coli* (11.5%) and *Enterococcus* (87%) contamination than those purchased during the winter (0 and 15% respectively). Animal feed purchased in the summer were 38 times more likely to be contaminated with *Enterococcus spp*. than animal feed purchased in the winter (p-value<0.001). There was also a significant association between being *Enterococcus* positive and being *E.coli* positive (p-value 0.0365). *Enterococcus spp.* positive samples were 8 times more likely to also be *E.coli* positive when compared to *Enterococcus spp.* negative samples. The overall sample size of 158 was sufficient to detect bacterial contamination of 10% or more with a power of 80%. Thus, the failure to isolate *Campylobacter* indicates that if

present, its prevalence is likely to be less than 10%. More intensive sampling would therefore be needed to determine its prevalence in feed.

3.5 Discussion:

The prevalence of *Salmonella* (0.6%), and *E. coli* (5.7%) in plant sourced retail animal feed were approximately 10 fold lower than those estimates obtained from the FDA-CVM 2003 survey (5% and 43% respectively). But, they were consisted with results of Crane et al, who reported that only 2% of incoming ingredients were contaminated with *Salmonella* and without meat meal, only 0.7%.[34] This study estimates the prevalence of *Enterococcus* contamination at approximately 51% while the FDA-CVM reported a prevalence of 91%. However, the relative contribution from each bacterial species is similar and the difference in magnitude may be due to differences in the population sampled by FDA-CVM when compared to this study. Samples for the FDA-CVM study were collected from commercial feed mills and on-farm mixers then pooled together prior to bacterial isolation from each batch. Thus, these were two very different populations being sampled. Samples collected in this study were from small-scale animal feed retail stores intended for hobby and small-scale livestock producers but feeds were purchased from commercial feed mills. Most of these samples were packaged in individual bags containing 50 lbs or less of feed. This distribution method differs significantly from that commonly employed on large-scale commercial livestock and poultry farms and it may result in much lower rates of bacterial contamination.

In this study, microbial contamination in the feed samples purchased in the summer was higher than those purchased in the winter. This may reflect a seasonal effect

related to weather and production, processing, and storage conditions; a one-time contamination; or a pattern of increasing contamination. Additional prospective research is needed to determine what the seasonal pattern of contamination is and to identify the risk factors associated with this pattern. Other important patterns affecting the prevalence of bacterial contamination of feed also need to be taken into consideration when developing the sampling frame for studies investigating the prevalence and distribution of bacterial contamination of feed. Jones *et al* reported there was no statistical difference in the *Salmonella* contamination rate of commercially manufactured animal feed samples collected in the spring when compared to samples collected in the summer but they found that the contamination rate was higher in samples collected on Friday when compared to samples collected on Monday or Wednesday.[90]

Jones *et al* also reported that feed samples (both mash and pellets) contaminated with *Salmonella* contained significantly higher *Enterobactericiae* counts (EC) than samples not contaminated with *Salmonella* and concluded that EC may provide some indication of the likelihood of *Salmonella* contamination in feed samples.[90] These results are consistent with this study in which all *Salmonella and E.coli* positive samples were also positive for *Enterococcus,* and the risk of *E.coli* contamination was 8 fold higher in those feed samples that were *Enterococcus* positive when compared to those samples what were *Enterococcus* negative. Thus, being *Enterococcus* positive may be an indicator of fecal contamination and contamination by potential pathogens such as *Salmonella*.

Enterococcus is normal gut flora for humans and many livestock species. It is widely disseminated in the environment and as demonstrated by the FDA-CVM studies

and this study, it can readily be isolated from animal feed. While it is not usually a human pathogen, it can cause infectious disease and ranks second to third in frequency among bacteria isolated from hospitalized patients. Enterococcal infections occur in hospitalized patients with a variety of underlying conditions and across a wide spectrum of severity of illness and immune modulation.[92] While the role of animal feed as a risk factor for exposure of humans to pathogenic strains of *Enterococcus* has not been established, its common prevalence in feed may indeed prove to have health implications for livestock and humans especially if feed is identified as a reservoir for antibiotic resistant strains of *Enterococcus*[82]. Recently, antibiotic resistant *Enterococcus* isolates were identified in US hospitals. Reports also indicate that antibiotic resistance genes from *Enterococcus* are capable of transferring to other bacterial species.[15] FDA-CVM survey reported that antibiotic resistant *Enterococcus* strains occur frequently in feedstuff, but they appear to have widely disseminated phenotypes that occur with equal rates in both animal and plant derived products. In addition, the inherently resistant *Enterococcus, Salmonella* or *E.coli* shed by livestock into litter or manure have the potential to harbor antibiotic-resistant plasmids that may be transferred from litter or manure-treated lands to feed and food derived from crops grown on such lands.[9,120]

This was a preliminary study to determine the magnitude of the problem with contamination of retail plant-based animal feed with enteric bacteria. The sample size was only sufficient to detect contamination at a level of 10% or greater and the sampling periods only included two seasons, summer and winter. A greater sample size with more frequent sampling intervals is necessary to clearly establish seasonal patterns of enteric bacterial contamination of retail plant-based feed. A prospective study design that follows

crops from harvest to farm delivery is necessary to identify risk factors from the field through to feed production continuum. Each of these study designs needs to be developed with consideration of the seasonal and weekly patterns of bacterial contamination that have been reported. Despite these limitations, results from this study and others in the literature demonstrate that microbial contamination in animal feed should be a great concern for commercial feed producers, retail distributors, livestock and poultry producers and the general public. Improvement in the safety of animal feed should include strengthening the surveillance of animal feed for bacterial contamination and integration of such surveillance with human foodborne disease surveillance systems.[17,82,101] In order to achieve the FDA-CVM goal of *Salmonella*-free feed and feed ingredients, a HACCP program needs to be instituted for the animal feed industry. A comprehensive feed and food safety approach will require both a "farm-to-fork" model and a "field-to-trough" model of risk assessment.

Table 1. Univariate analysis of enteric pathogens contamination in plant protein for animal feed and milk replacers in Maryland and Northern Virginia, sampled in 2002. (N=158).

	N	*Entero-Coccus*	*E.coli*	*Salmo-nella*	*Campylo-bacter*
Winter batch	80	12 (15%)*	0*	0	0
Summer batch	78	68 (87%)	9 (11.5%)	1 (1.3%)	0
Total	158	80 (51%)	9 (5.7%)	1 (0.6%)	0

* Statistically significant difference at $\alpha = 0.05$

Table 2: Summary results of *E.coli* and *Enterococcus* contamination of vegetable protein collected from Maryland and Northern Virginia in 2002.

Meal types	*E.coli* Prev (%)	*Entero* Prev (%)
Corn	6 (3/52)	52 (27/52)
Peanut	8 (1/12)	42 (5/12)
Sunflower*	10 (2/10)	40 (8/20)
Cotton Seed	0 (0/12)	50 (6/12)
Soy bean	0 (0/12)	50 (6/12)
Saffflower	0 (0/10)	40 (4/10)

* One sample tested positive for *Salmonella*.

4. Chapter III: Airflow in Broiler Houses as a Risk Factor for Growth of Enteric Pathogens

4.1 Abstract

Salmonella is one the most important foodborne diseases worldwide and *Salmonella* control has become an important objective for the poultry industry from both public health and economic perspectives. Increased water parameters (moisture, and water activity) in litter may create favorable conditions for bacterial multiplication. A cross-sectional study was conducted to assess the association between airflow within a poultry house and litter *Salmonella* and fecal coliform loads. Five different commercial university broiler farms at two different geographical locations were sampled. Overall *Salmonella* prevalence by the drag swab (DS) method was 50% (18/36) of swabs, representing 4 of 5 houses sampled. Individual litter samples had a *Salmonella* prevalence of 33.3 % (12/36) representing 3 of the 5 houses sampled. In all five houses, airflow velocities at three feet (91.2cm) above the litter surface were greater than the airflow measured at three inches (7.6cm) above the litter surface. On Farms1, 2,3 and 5, regions within the poultry house with reduced airflow velocity were associated with regions having increased fecal coliform counts. This association was not observed at Farm 4.

The Friedman two-way analysis of variance by rank found a significant association between regions of reduced airflow within a poultry house and regions of increased coliform and *Salmonella* contamination. Even within tunnel ventilated houses, supplemental ventilation may be necessary in those areas of reduced airflow to achieve a

target of 1.5 mph at 3 inches above the litter surface as the minimum rate of airflow within the house. This modest increase in airflow can significantly reduce the level of fecal coliform and *Salmonella* contamination and improve both poultry health and productivity and poultry product safety.

4.2 Introduction

Salmonellosis is the most frequently reported foodborne illness in the United States, and is the second most common foodborne illness worldwide[1]. Controlling *Salmonella* has thus become an important objective for the poultry industry from both a public health and economic perspectives. *Salmonella* can be found in virtually every part of the world and is carried by an extremely wide variety of hosts. Effective *Salmonella* control at the farm especially in broiler litter and layer manure could lower *Salmonella* contamination in poultry products.[21,79,108] High water parameters (moisture which can be defined as diffuse wetness that can be felt as vapor in the atmosphere or condensed liquid on the surfaces of objects; dampness, and water activity (Aw) (or equilibrium relative humidity %ERH) measures the vapor pressure generated by the moisture present in a hygroscopic product reflects the active part of moisture content or the part which, under normal circumstances, can be exchanged between the product and its environment) in litter facilitate the multiplication of enteric pathogens such as *Salmonella* and *E. coli.* A variety of studies have shown that Aw greater than 0.90 at broiler litter surface were associated with increased *Salmonella* prevalence in poultry houses and on carcasses of birds processed from these houses.[52,108] These studies conclude that lower Aw levels at the litter surface are associated with lower *Salmonella* loads on carcasses. Therefore, the transmission of *Salmonella* from the farm to the processing plant and potentially to marketed carcasses may be diminished and controlled by implementing management strategies that reduce bacterial loads in the production environment.

Reduction of moisture within a poultry house can be achieved through adequate ventilation. In fact, one of the goals of tunnel ventilation systems is to reduce moisture in

the poultry house environment.[24,173] However, an association between rate and pattern of airflow within the poultry house and distribution of litter bacterial load has not been established. Increased airflow, especially in those regions of the house where the airflow may be relatively stagnant even with tunnel ventilation, can lower Aw levels at litter surface throughout the house. This may also indirectly reduce bacterial loads in litter and consequently reduce bacterial loads in the birds at processing. Identification of regions within the poultry house with increased *Salmonella* and fecal coliform multiplication can lead to the development of interventions, such as supplemental ventilation, to control bacterial growth in those "hot spots". Kingston and colleagues have shown that such hygienic environments are closely associated with reduced prime costs of broiler production, and *Salmonella*-negative carcasses at processing.[21,95,108,129] This will enhance both poultry health, productivity and product food safety.

The objectives of this study are:

- To study the airflow pattern inside broiler houses
- To evaluate the relationship between airflow and the growth pattern of *Salmonella* and fecal coliforms (FC) in broiler house litter

The hypotheses of this study are:

- Litter samples from the region of minimum airflow within a poultry house is associated with increased *Salmonella* and FC bacterial loads when compared to litter samples from the region of maximum airflow within the same poultry house.
- The correlation coefficient for airflow at a level of 3 inches above the litter and FC count is significantly greater than the correlation coefficient for airflow at level of 3 feet above the litter surface and FC count.

4.3 Materials and Methods

Study Design: A cross-sectional study approach was used to assess the association between airflow within a poultry house and litter *Salmonella* and FC loads.

Five commercial broiler houses from three different commercial poultry operations located on university farms in Eastern and Southern parts of the United States were visited on single occasions during the final week of a six-week grow-out period. Each house was sampled by six-drag swabs (DS), which were collected from the left, center and right sections of the poultry houses. After dragging, the swabs were returned to the transport containers, labeled with date, farm number, and region dragged, and placed in an insulated foam box. Airflow within the poultry house was monitored using a sampling grid technique where 30 evenly spaced intervals of the house were marked. Airflow was then measured at a height of 3 inches (7.6cm) above the litter surface and 3 feet (91.4cm) above the litter surface. Airflow patterns over each sampling site were measured using a digital Hygro-thermometer, anemometer, data-logging instrument (Pacer Industries, Inc., Chippewa Falls, WI) and digital thermo wind meter (Spectrum technologies, Inc., Plainfield, IL) according to the manufacturer's instructions. Each air velocity reading represented the maximum airflow at a particular location during a 30-s interval. From the collected airflow data, 2 maximum, 2 median, and 2 minimum airflow locations were marked for litter sample collection.

A 25-gram litter sample was collected at each of the 6 designated locations. Litter samples were transferred onsite into 50 ml centrifuge tubes containing 25 ml of 2% buffered peptone water (BPW). Tubes were labeled with the date, farm number, and site

number and sealed. Samples were placed in a styrofoam shipping box containing dry ice and sent to the laboratory via overnight courier. Drag swab and litter samples were processed within 24 hrs of collection. All litter samples were processed according to Mallinson *et al*[107]. The frozen litter samples were thawed quickly in a hot water bath (41°C). The litter was weighed and transferred to 225 ml BPW, thoroughly shaken for about 10 minutes, then filtered using a stomacher bag (Fisher brand filtra bag). A 45 ml aliquot of the filtrate was placed into a 50ml plastic tube and frozen at -70°C. The remaining filtrate was placed in a 400ml plastic bottle and placed in a 37°C incubator overnight.

***Salmonella* Quantification:**

Litter samples were pre-screened for *Salmonella* quantification as previously described. *Salmonella* screening was performed by qualitatively testing for this organism using filtrate. After primary and selective enrichment in BPW and Rappaport Vassiliatis (RV) broth, respectively, all samples were placed on Miller-Mallinson (MM) agar and incubated at 37°C. Plates were read after 24 and 48 hrs of incubation. Suspected colonies were confirmed by biochemical test with Triple Sugar Iron (TSI) and Lysine Iron (LIA) agar. Positive litter samples were quantified with a three tubes serial dilution technique using the most probable number (MPN) calculation by FDA- Bacterial Analytical Manual (BAM) method.[7]

Fecal Coliform Quantification:

FC quantification was performed by thawing 45-ml aliquot suspension of litter, which was serially diluted as 200μl, 20μl, 2 μl and 0.2 μl and filter through microbial

monitor (Schleicher & Schuell MicroScience, Inc. USA Riviera Beach, FL) for each dilution. 2ml M-fc medium with rosolic acid, (Millipore Corp., Bedford, MA) for FC was put on each monitor and incubated at 41°C for 24 hrs. Typical blue FC colonies were counted for quantification from each dilution and colony forming units (cfu) per 10 grams of litter sample was calculated based on number of colonies, dilution ratio, and litter sample weight.

Statistical Analysis:

Statistical Software (Statistix-8, Tallahassee, FL) was used to analyze the data. Raw and transformed data were tested for normality using the Shapiro-Wilkes test[39]. Since farms differed by age of litter, age of house, temperature and humidity at the time of sampling, and type and age of ventilation system, analysis was conducted on samples from each house separately. Paired t-test was used to compare FC counts between low and high airflow samples for each separate farm[39]. Fisher's exact test was used to compare binary outcomes and to estimate odds ratios[39]. Spearman Rank Correlation coefficient test was used to compare correlation coefficient of airflow at 3 inches above litter and FC counts with airflow at 3 feet above litter and FC counts[39]. The Friedman two-way analysis of variance by rank was used to measure the effect of different airflow levels to the FC coliform counts in the broiler litter samples[39].

4.4 Results

The results of descriptive analysis of variables of sample (airflow at 3", airflow at 3', *Salmonella* MPN#, and fecal coliform cfu counts for each house are summarized in

Tables 3,4 and 5. *Salmonella* and FC counts were not normally distributed by Shapiro-Wilkes test. These data were then transformed using the natural log transformation. The results of the Shapiro-Wilkes test on the transformed data indicate that the transformed data were normally distributed (p-value 0.2680 for *Salmonella* and p-value 0.8452 for FC counts).

Overall *Salmonella* prevalence by DS method was 50% (15/30), and by litter samples, 33.3 % (10/30). (Table 6) In all five houses, airflow velocities at three feet above the litter were greater than at three inches above the litter. The correlation coefficient for airflow at 3 inches above the litter and the fecal coliform count is significantly greater than the correlation coefficient for airflow at 3 ft above the litter surface and coliform count for Farms 1, 4 and 5 (Table 7).

There was an inverse association between airflow and FC counts in Farms 1, 2 and 3. The resulting relationship was a 3-4-fold reduction in FC count at those sites in which the airflow exceeded the median for the house when compared to those sites where the airflow was below the house median velocity (Table 8). A summary of the relationship between airflow and FC counts for each farm is shown in Figures 1-5. Farms 2 and 3 had a statistically significant difference between FC counts from low airflow litter sampling sites and FC counts from high airflow litter sampling sites with p-value 0.0276 for Farm 2 and p-value 0.0111 for Farm 3. There was no statistically significant difference between FC counts and air velocity in Farms 1, 4 and 5. Also, there was no statistically significant difference between different levels of airflow to FC coliform counts between broiler houses from southern states (p-value 0.097).

The Friedman's two-way analysis of variance by rank found an association between minimum airflow and elevated FC rank when compared to maximum airflow and FC rank. This difference was statistically significant at α level 0.10 (p-value 0.0578). (Table 9)

4.5 Discussion

As reported by De Rezende *et.al*, the DS method was more reliable and sensitive for qualitatively determining the *Salmonella* status of a house than litter samples[53]. The high percentage of negative litter culture results compared to the DS results suggests that *Salmonella spp*. is not uniformly distributed throughout the litter surface. At moderate relative humidity, there is a significant association between regions of reduced airflow within a poultry house and regions of increased coliform and *Salmonella* contamination. Samples collected from farms where relative humidity was 90-95% failed to show significant association between airflow and bacterial load. These houses also had significantly increased bacterial load overall when compared to farms sampled where relative humidity was less than 50%. Farms 1, 2 and 3 are from the Mid-Atlantic regions of the United States. At the time of sampling, relative humidity on these farms was approximately 43% and outdoor temperature was 80°F, which was much lower than the relative humidity and temperature at the time of sampling on Farms 4 and 5 (90-95% and 88°F respectively) located in the southern United States. In addition, age of the litter may have been an important risk factor for the level of bacterial contamination observed in Farms 4 and 5. Houses from these farms had new litter placed just prior to the arrival of the current flock of birds, while houses from Farms 1, 2, and 3 had cycled previous flocks

through the litter before the flock that was sampled for this study. Corrier *et al* reported that used litter from floor pens of adult broilers had a competitive exclusion effect on *Salmonella* colonization of broiler chicks.[28] Other factors need to be considered for future investigations are litter amendments such as humidity, pH of litter and antibiotics usages. Also, the size of broiler house, number of fans installed, ventilation type, and bird capacity are important to consider for airflow movement in broiler houses.

Airflow results indicated that areas exposed to higher ventilation rate than 3.5 miles per hour (mph) were associated with lower humidity and FC population. The low prevalence of *Salmonella*-positive litter samples precluded analysis of an association between airflow and *Salmonella* load. Although ventilation practices varied widely between farms (number of placement, static pressure, running time of fans, and curtain setting), a correlation seemed to exist between the rate of airflow over a specific litter location and its relative humidity. In contrast to houses that were ventilated by wind and propeller fans, tunnel ventilated houses had a greater and unvarying flow of air (3.3 to 4.8 mph) over the entire litter surface in the brooder chamber. It was also noted that, velocity at 3 inches above litter surface was lower than at 3 feet above the surface even in empty broiler houses. It can be expected that air velocity at the level of the birds is hindered even more when market-age birds are present. Lacy *et al*. reported that tunnel ventilation had advantages of reduction of heat stress related mortality and improved feed conversion and these advantage could also be serve as an effective means of removing excessive moisture from litter, thereby helping to suppress *Salmonella* and *E.coli* levels and risks.[99] In naturally ventilated houses, an attractive alternative to sidewall propeller fans, ceiling fans or perhaps even baffles may provide more homogenous airflow coverage over the

litter bed[19]. This study provided more information that relative humidity and airflow over the litter surface represent potentially important poultry house parameters that need to be monitored to ensure a safe and healthy environment for broiler performance by taking appropriate measures which could involve improving the uniformity of moderate airflow rates of at least 1.5 mph in lower humidity regions and at least 3.5 mph in higher humidity regions. While avoiding establishment of undesirable dry dusty conditions, the corrective management practices suggested here might likely produce those litter parameters associated with reduced risks of fecal coliform and *Salmonella* contamination.

Table 3: Summary results of descriptive analysis of airflow* at 3 inches and 3 feet above litter surface for 5 commercial broiler farms.

Farm#	Airflow at	Mean	Minimum	Median	Maximum	SE
1	3 inches	1.4	0.0	1.5	3.3	0.11
1	3 ft	1.7	0.4	1.5	4.6	0.14
2	3 inches	0.7	0.0	0.7	1.3	0.05
2	3 ft	0.9	0.0	0.8	1.5	0.05
3	3 inches	2.9	0.4	2.0	7.3	0.36
3	3 ft	4.1	1.3	3.3	9.8	0.42
4	3 inches	3.3	1.2	3.1	7.0	0.20
4	3 ft	6.0	2.1	6.2	8.0	0.24
5	3 inches	4.4	2.0	4.6	7.0	0.22
5	3 ft	5.9	1.2	6.3	7.7	0.28

*In all 5 farms, airflow is greater at 3 feet above the litter surface in comparison with airflow at 3 inches above the litter surface.

Table 4: Summary results of descriptive analysis of *Salmonella* MPN for 5 commercial broiler farms.

Farm#	Mean	Minimum	Median	Maximum	SE
1	0.0	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0	0.0
3	1.0	0.0	0.3	5.0	0.8
4	229.3	0.0	0.0	889.0	154.1
5	$27x10^3$	0.0	764.5	$125\ x10^3$	$20\ x10^3$

Table 5: Summary results of descriptive analysis of FC cfu count for 5 commercial broiler farms.

Farm#	Mean	Minimum	Median	Maximum	SE
1	8.0	1.6	3.8	22.5	3.42
2	1.9	0.3	1.0	4.6	0.76
3	1.7	0.4	1.4	4.0	0.57
4	6.8	0.7	6.2	15.2	2.03
5	63.7	3.5	17.6	285.0	44.58

Table 6: Summary Results for detection of *Salmonella* on five commercial broiler farms by drag swab and litter samples (N= 60)

Farm #	*Salmonella* Isolation %	
	Drag swab	Litter sample
1	0 (0/6)	0 (0/6)
2	33.3 (2/6)	0 (0/6)
3	66.7 (4/6)	50 (3/6)
4	83.3 (5/6)	33.3 (2/6)
5	66.7 (4/6)	83.3 (5/6)
Total	50 (15/30)	33.3 (10/30)

Table 7: Summary analysis of correlation coefficient of airflow at 3 inches and airflow at 3 feet above litter with FC cfu counts for each farm.

Farm #	airflow correlated to FC counts	Correlation coefficient
1	3 inches	-0.4119
1	3 ft	-0.8117
2	3 inches	-0.8117
2	3 ft	-0.6000
3	3 inches	-0.8117
3	3 ft	-0.8117
4	3 inches	0.2571
4	3 ft	-0.2571
5	3 inches	-0.3189
5	3 ft	-0.5429

Table 8: Results of association between airflow and FC counts in each farm in the Eastern United States.

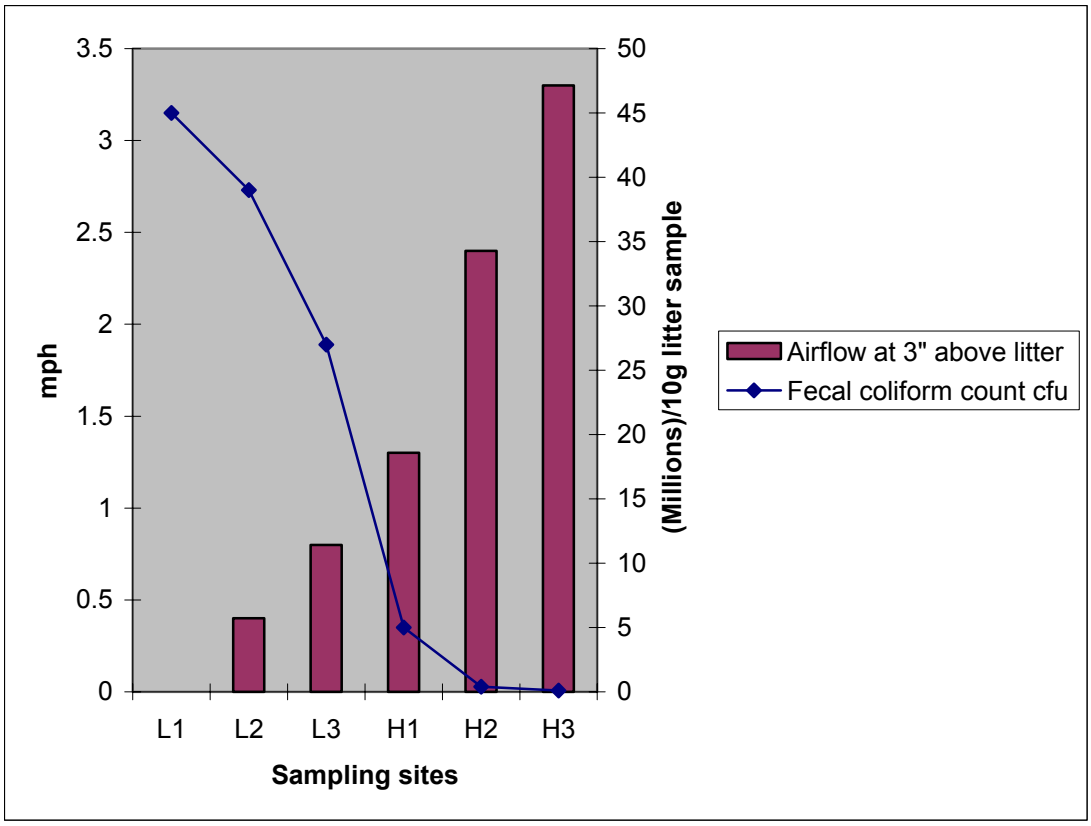
Farm #	Median Airflow (mph)	Median FC counts (millions)/10g	OR	95% C.I
1	2.5	2.5	3	0.8 – 100.9
2	0.52	0.7	4	2.6 – 111.4
3	2.5	0.8	4	2.6 – 111.4

Table 9: Results of analysis for FC counts at minimum airflow and maximum airflow from 5 commercial broiler farms by the Friedman two-way nonparametric analysis of variances.

	Mean Sample			
Variables	Rank	Size	Friedman Statistic	P-value,
FC counts at minimum airflow	1.80	10		
			3.6000	0.0578*
FC counts at maximum airflow	1.20	10		
Degrees of Freedom		1		

* Statistically significant at $\alpha = 0.1$

Figure 1: Summary result of relationship between airflow at 3inches above litter surface and fecal coliform cfu counts for Farm 1.



L = Low airflow

H = High airflow

Figure 2: Summary result of relationship between airflow at 3 inches above litter surface and fecal coliform cfu count for Farm 2.

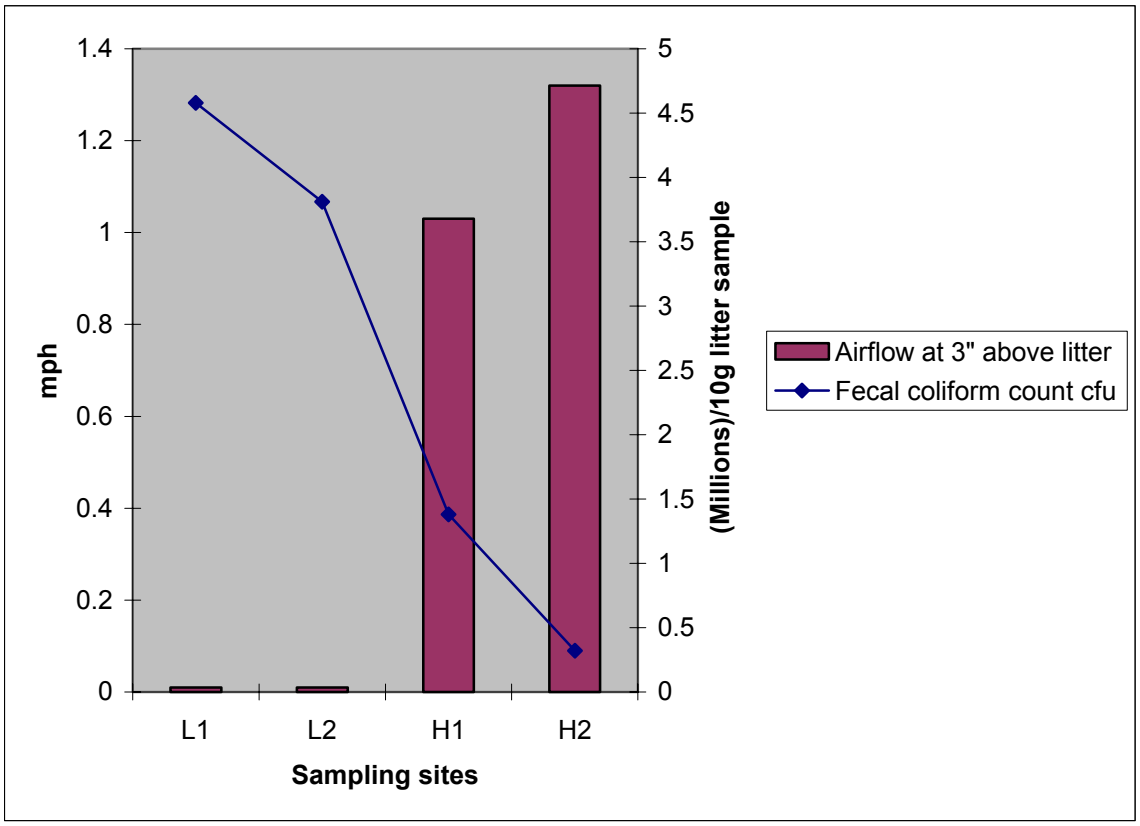


Figure 3: Summary result of relationship between airflow at 3 inches above litter surface and fecal coliform cfu count for Farm 3.

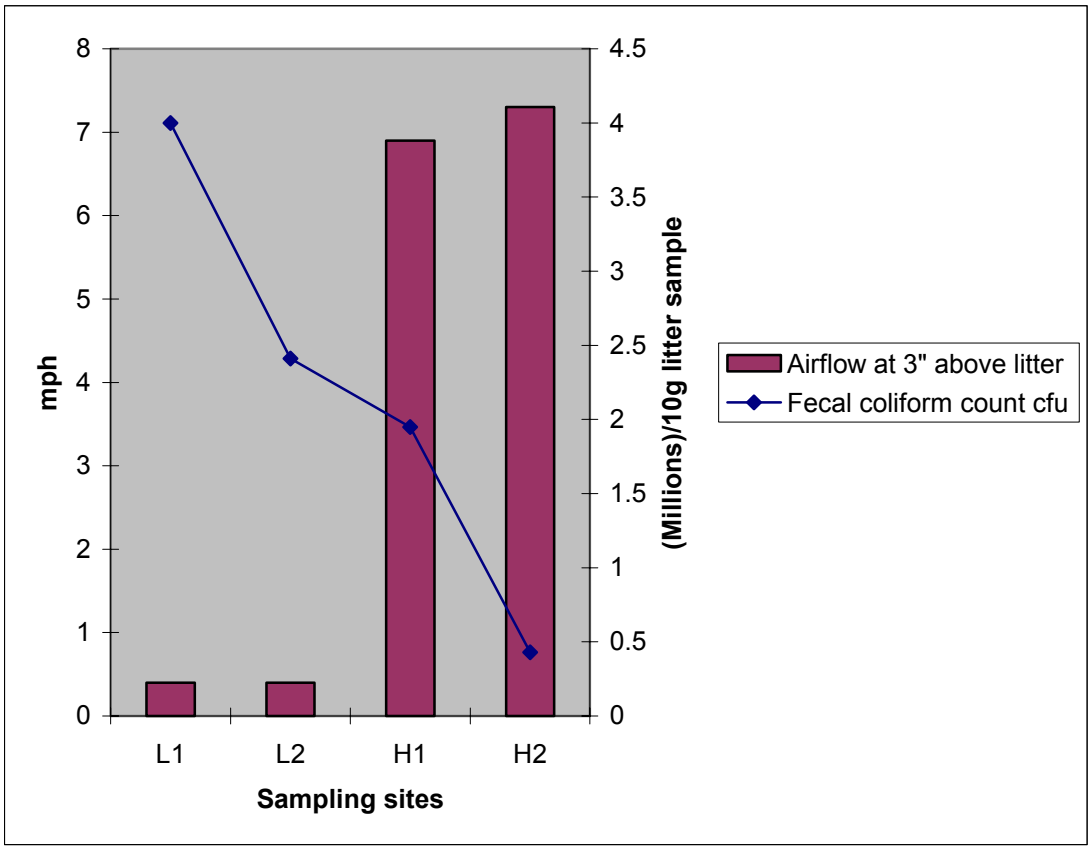


Figure 4: Summary result of relationship between airflow at 3 inches above litter surface and fecal coliform cfu count for Farm 4.

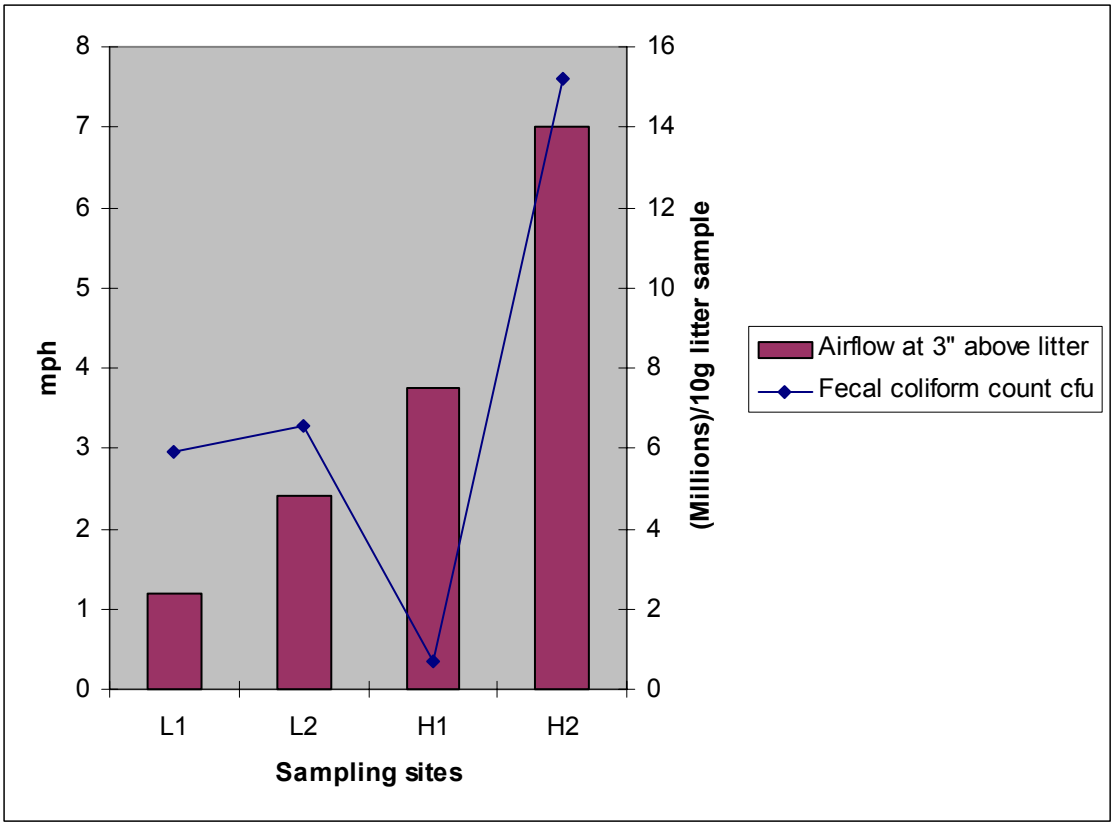
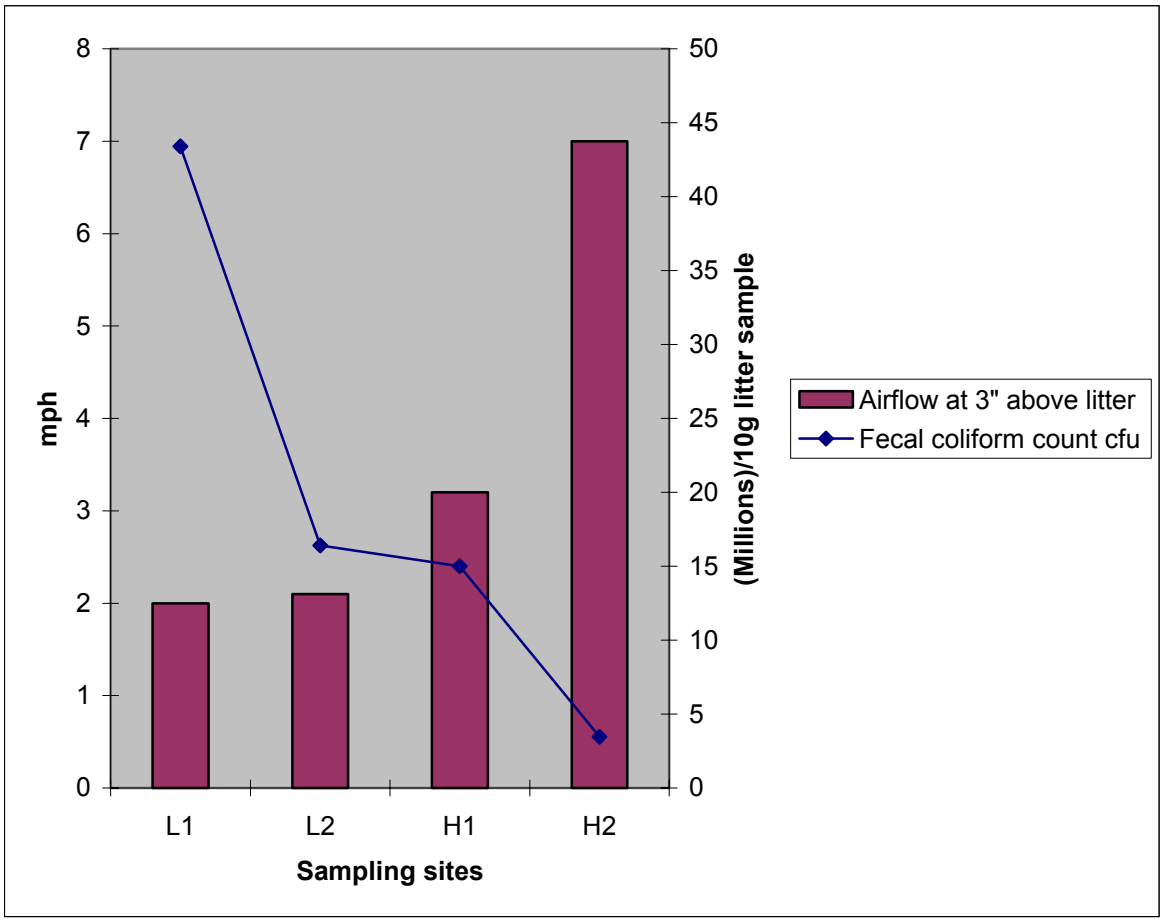


Figure 5: Summary result of relationship between airflow at 3 inches above litter surface and fecal coliform cfu count for Farm 5.



5. Chapter IV: Estimation of Sensitivity and Specificity for a PCR Based Diagnostic Test for Detection of *Salmonella* Contamination in Raw Poultry Products

5.1 Abstract

Salmonella spp. are the leading cause of foodborne illness worldwide. Conventional culture techniques for the detection of *Salmonella spp.* are generally labor intensive and time-consuming. More rapid detection methods have been developed over the past few years. However, standard methods for sample handling and preparation have not been established and limited data is available in the literature on the validity of these tests for the detection of *Salmonella* contamination in naturally contaminated retail meat samples. Using standard culture techniques as a gold standard for *Salmonella* detection in naturally contaminated raw poultry products, the sensitivity and specificity of a PCR detection method was determined. Chicken meat samples were pre-enriched in buffered peptone water (BPW) and *Salmonella* specific primers ST 11 and ST 15 were used to amplify a 429 bp region specific to all *Salmonella spp*. There was a significant decrease in the sensitivity of the PCR test when BPW enrichment alone (85%) was used compared to the sensitivity achieved after both BPW enrichment and selective enrichment 100 % (p-value<0.001). There was no significant difference in the test specificity for any of the three detection methods. A minimum of 12 hours pre-enrichment was required for detection of *Salmonella* by PCR at a limit of 100 cfu. No detectable amplification product was detected in those samples testing negative by culture methods. Detection of *Salmonella* contamination at a level of greater or equal to 100 cfu by PCR can be

conducted with adequate sensitivity and specificity within 24 hours after both pre-enrichment in BPW and selective enrichment with TT-H in naturally contaminated chicken meat samples.

5.2 Introduction

Salmonella species are a major cause of foodborne illness in humans worldwide.[57,148,171] Contaminated poultry products have been identified as the principal sources of *Salmonella*[139,140,159] leading to foodborne illness in humans. Handling of raw poultry carcasses and products and consumption of undercooked poultry meat are the main causes of infection.[26,127] Conventional culture methods to detect *Salmonella spp*. are generally labor and time-consuming processes, requiring a minimum of 4 to 6 days. Culture methods have also been reported to show poor sensitivity for low-level contamination in samples. Standardized rapid, sensitive and specific detection techniques for *Salmonella spp*. are important for enhancing bird health and productivity, poultry product quality and consumer confidence and as a tool for public health officials seeking to reduce the economic and health impact of foodborne illness in humans.[2,22,110]

Rapid methods such as RT-PCR or ELISA have been developed to overcome this problem, but the current literature does not provide sufficient data to validate the sensitivity and specificity of these methods for the detection of *Salmonella* in naturally contaminated retail food samples. In addition, comparisons of study results are often not possible since the techniques for sample handling and enrichment vary between studies. Thus, conventional cultural methods are still widely used for the detection of *Salmonella spp.* Research to standardize and validate *Salmonella* detection methodologies is necessary because further control of these pathogens will depend increasingly on the availability of rapid and precise diagnostic tests for monitoring primary animal products, food processing plants, and ready-to-eat food products.[2,22,26]

Most of the studies of *Salmonella* detection methods have used artificially contaminated or spiked samples.[1] However, the physiological state of bacteria from artificially contaminated samples does not reflect the physiological state of bacteria from naturally contaminated food samples, which may have been exposed to a variety of unfavorable conditions or suffered some degree of injury while in transport or processing.[74,144] In addition, the sensitivity of a test when applied to an artificially dosed sample may differ significantly from its ability to detect the much smaller bacterial loads that can be anticipated in a sample with naturally occurring bacterial contamination.

The polymerase chain reaction (PCR) method is one of the most promising of the rapid microbiological methods for the detection and identification of bacteria in a wide variety of samples. The high sensitivity and specificity of the PCR method can be an attractive means to achieve this goal. The PCR method is widely used in clinical and research environments[102]. However reports of its usage in food products are still limited.

Since the direct application of PCR to complex substrates often results in an absence of detectable amplification products or poor sensitivity,[46] bacterial DNA extraction must be performed to avoid inhibition of the PCR by food components. The PCR is based on nucleic acid amplification and essentially consists of sample preparation to extract the target nucleic acid, amplification of the target DNA, and analysis of the amplification products. The modified procedure of Van Lith and Aarts[162] was used and which the isolation of DNA by mere heat lysis without chemical or enzymatic lysis of bacterial cells was used.

The objectives of this study were:

- To estimate the effect of different enrichment protocols on the sensitivity and specificity of PCR to detect *Salmonella* in naturally contaminated meat samples when compared to culture.
- To estimate the minimum pre-enrichment period and bacterial load that can be detected by PCR.

Hypotheses:

1) Pre-enrichment causes no significant difference in sensitivity and specificity of PCR when compared to culture.

2) Pre-enrichment causes no significant difference in concordance between PCR and culture.

3) PCR and conventional culture have a concordance rate of $\geq$ 95%.

5.3 Materials and Methods

Sample collection:

A total of 90 chicken meat samples were obtained from 14 grocery stores throughout Maryland. From each sample, two 25 g sub-samples were collected to detect and isolate *Salmonella* by conventional culture [107] and PCR.[74]

Pre-treatment of sample:

Each 25g sub-sample was pre-enriched at 37°C in 225 ml of buffered peptone water (Becton and Dickinson, USA) in a shaking incubator overnight. These pre-enriched samples were then used for the PCR assay and for conventional culture.

For the PCR assay 1 ml of pre-enriched suspension was transferred to eppendorf tubes. For culture technique, 0.1ml of suspension was transferred to 9.9 ml of Rappaport Vassiliadis (RV) medium (Becton and Dickinson, U.S.A) and 0.5 ml of suspension was transferred to 10 ml of Tetrathionate-Hajna (TT-H) and incubated at 41.5°C overnight. One loopful of broth was streaked onto Xylose Lysine Tergitol-4 (XLT-4) agar (Becton and Dickinson, U.S.A) and the plates were incubated at 37°C for up to 72 hours. Presumptively positive samples were tested with triple sugar iron (TSI), lysine iron agar (LIA) for biochemical reaction and later confirmed with Serum "O" and "H" (Murex Diagnostics, Kent, UK; Behring, Germany) by precipitation reaction.

Preparation for PCR:

DNA samples were extracted at 3 stages from chicken meat samples: without pre-enrichment; after 24 hrs pre-enrichment with BPW; and after 24 hrs pre-enrichment and 24 hrs selective enrichment with TT-H or RV. Template DNA was extracted from poultry meat products by a modification of the rapid lysis method of Van Lith and Aarts.[162] 1-ml suspension samples were low-speed centrifuged (1,000Xg, 2 min) to allow meat particles to precipitate. The supernatant was then centrifuged (13,800Xg, 10 min), supernatant removed, and the pellet washed twice in a sterile buffered physiological saline solution followed by centrifugation of the cells (13,800Xg 10 min). The pellet was then re-suspended in 100 µl of sterile milli Q water and heated at 95°C for 10 min. The cell lysate was immediately placed on ice. Following the extraction procedure, 2 µl of the extraction products containing the DNA was tested by PCR-based assays.[171]

The extracted DNA (2 µl) was added to a reaction mixture (48 µl) consisting of TaKaRa Ex Taq 0.5µl, 10X Ex Taq Buffer 10µl, dNTP mixture (2.5mM each) 8µl, primer 1 (ST-11); sequence (AGC CAA CCA TTG CTA AAT TGG CGC A) 1.0 µM, primer 2 (ST-15); sequence (GGT AGA AAT TCC CAG CGG GTA CTG) 1.0 µM and sterilized distilled water 37.5µl. Each tube was put in a thermal cycler. Samples were denatured at 94°C for 2 min.; thirty-five cycles of amplification were run at 95°C for 30 seconds, at 60°C for 30 seconds, and at 72°C for 30 seconds. The reaction was completed by a final 10 minutes extension at 72°C. Aliquots of amplification products were separated on 0.5% agarose gel in 0.5X Tris Borate Ethylenediaminetetraacetic acid (EDTA) buffer and visualized by ethidium bromide staining and UV transillumination.

Aliquots of positive samples tested with PCR were saved before pre-enrichment at -70°C. From these samples, five samples were later used to determine the minimum time requirement for pre-enrichment with BPW. DNA was extracted from aliquots every two hours until positive results were found on gel. Pre-enriched samples were serially diluted and plated on XLT-4 agar to quantify colony forming units (cfu) of *Salmonella* in samples and also, extracted DNA to determine the minimum cfu which can be detected by PCR.

Calculation of Sensitivity and Specificity

The sensitivity of the PCR test (Se) is the proportion of culture positive samples that test positive by PCR where culture test was used as a gold standard.[48] It is described statistically as the conditional probability of testing positive given that the sample is positive culture [p (PCRT+/ Cult T+)]. The relative agreement for these 2 tests beyond

chance, Kappa value was also calculated.[48] All hypotheses were tested with Fisher's exact tests[39] for a statistical significant difference between these two tests by using statistical software (Statistix-8, Tallahassee, FL).

5.4 Results

Of the 180 chicken samples, *Salmonella* was detected from 35 (19%) with the culture method, after pre-enrichment with BPW and selective enrichment with RV broth, and 42 (23%) with the culture method using pre-enrichment with BPW and selective enrichment with TT-H (Table 10). 33 samples tested positive for *Salmonella spp.* with specific primers by PCR after pre-enrichment in buffered peptone water. 42 positive samples were detected by PCR using DNA from the selective enrichment in TT-H media and 35 positive samples by PCR using DNA from the selective enrichment in RV broth (Table 10). No sample that tested negative with culture tested positive on PCR.

The results of sensitivity, specificity and kappa value calculation for the PCR test with different DNA extraction methods compared to culture are detailed in Table 11. Comparison of sensitivity and kappa value for PCR tests with different enrichment methods are shown in Figure 6. The results of requirement for pre-enrichment time are shown in Table 12. Samples without pre-enrichment were not detected by PCR. The earliest sample tested positive after 8 hours pre-enrichment. It had 128 *Salmonella* cfu. Samples with 16 and 68 cfu were also tested with PCR and yielded negative results.

5.5 Discussion

The PCR test for the detection of *Salmonella* spp. in chicken meat may be limited by the presence of substances that inhibit the assay. In this study, experiments using naturally contaminated or artificially challenged samples without pre-enrichment failed to yield the 429-bp by PCR. This result was consistent with the study reported by Gouws and colleagues.[74] A single 429-bp DNA was detected from artificially and naturally contaminated samples by the *Salmonella* specific ST 11 and ST 15 primers after pre-enrichment with BPW with or without selective enrichment[74]. Thus, the combination of 8 hrs pre-enrichment for PCR has the advantage of enhancing the sensitivity of the assay from 10^5 to 10^7 cfu/ml *Salmonella* and also reducing the negative influence of the meat sample.[144] Nonviable cells will not be able to grow during the pre-enrichment step, therefore also reducing the risk of false positive results. Since direct application of PCR to chicken samples and other food products usually results in no amplification, DNA extraction was performed to avoid inhibition of the PCR by food components. The combination of centrifugation and washing can enhance the sensitivity of the PCR method.[159] The extraction method in our study was reliable for artificially[74] and naturally contaminated chicken samples and no false-positive results were obtained.

The positive samples in this study required a minimum of 8 hrs pre-enrichment before they could be detected by PCR. Other literature indicates that bacteria that are naturally present in food samples usually have a reduced viability due to the prolonged exposure to unfavorable conditions such as high salt concentrations, unfavorable pH levels, freezing, and heating. According to Soumet *et al*., the detection of low-level *Salmonella* contamination in processed food products will probably require a much

longer incubation period in pre-enrichment broth.[144] Consistent with our findings, these authors advise that incubation for at least 10 hrs is necessary for detection of *Salmonella spp*. by PCR. The complex composition of food materials can hinder the PCR and lower its sensitivity. Moreover, the level of bacterial contamination of food products directly after processing is usually lower than in artificially contaminated samples where pre-enrichment may require a much longer incubation period before PCR can be used to detect low levels of *Salmonella* contamination.[1]

The use of selective enrichment RV and TT-H for suppression of bacteria other than *Salmonella* has been the subject of much discussion in the scientific literature. A significant finding from this study was the increased sensitivity found for culture and PCR when selective enrichment in TT-H was used rather than RV. It has been hypothesized that alkaline conditions are more favorable for *Salmonella* growth than RV's acidic pH. In this study, both PCR and bacterial culture demonstrated increased sensitivity when pre-enrichment with BPW was followed with selective enrichment in TT-H. *Salmonella* detection by PCR assays of poultry meat samples should be standardized to include a minimum 12 hrs BPW pre-enrichment and a minimum 8 hrs TT-H selective enrichment for maximum sensitivity. Under this condition, PCR was proven to be as sensitive as bacterial culture while providing results within 16 to 24 hours. Further investigations are, however, required to determine the most sensitive PCR protocols for environmental samples such as litter, feces, or feed.

Table 10: Results of *Salmonella* prevalence from poultry meat samples tested by culture and PCR after various enrichment steps*.

Tests	Prevalence
Culture test BPW + RV	35 / 180 (19%)
Culture test BPW + TT-H	42 / 180 (23%)
PCR test without enrichment	0 / 180 (0%)
PCR test for pre-enrichment with BPW	33 / 180 (18%)
PCR test for BPW + RV	35 / 180 (19%)
PCR test for BPW + TT-H	42 / 180 (23%)

*All PCR positive samples were also tested positive with culture test.

Table 11: Results of *Salmonella* prevalence, sensitivity, specificity, and Kappa value for PCR using DNA extracted at different stages of enrichment.

PCR Tests	Prevalence (%)	Sensitivity	Specificity	Kappa
No pre-enrichment	0.0	0.0	100	0.0
BPW	18	78.57	100	0.85
BPW+RV	19	83.33	100	0.89
BPW+TT-H	23	100	100	1.0

Table 12: Determination of the minimum time required to pre-enrichment with BPW to detect a positive with PCR.

Pre-enriched Time	Test results (Test positive/ Sample Tested)	Cumulative positive (%)
2	0/5	0
4	0/5	0
6	0/5	0
8	2/5	40
10	0/5	40
12	1/5	60
14	0/5	60
16	1/5	80
18	1/5	100

Figure 6: Comparison of sensitivity and Kappa value for PCR tests with different enrichment methods.

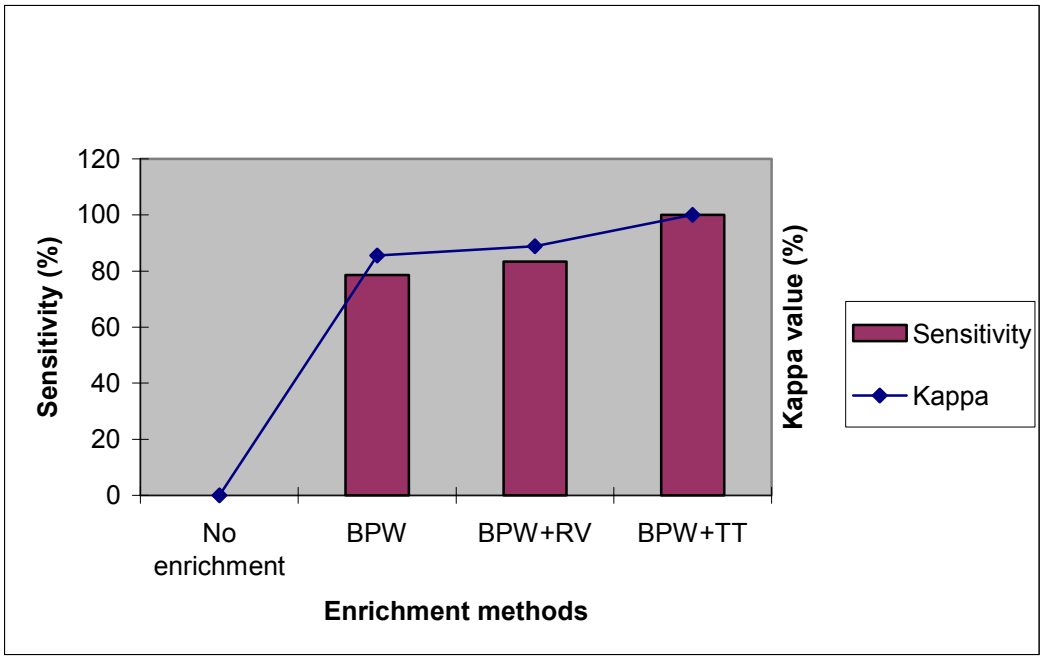
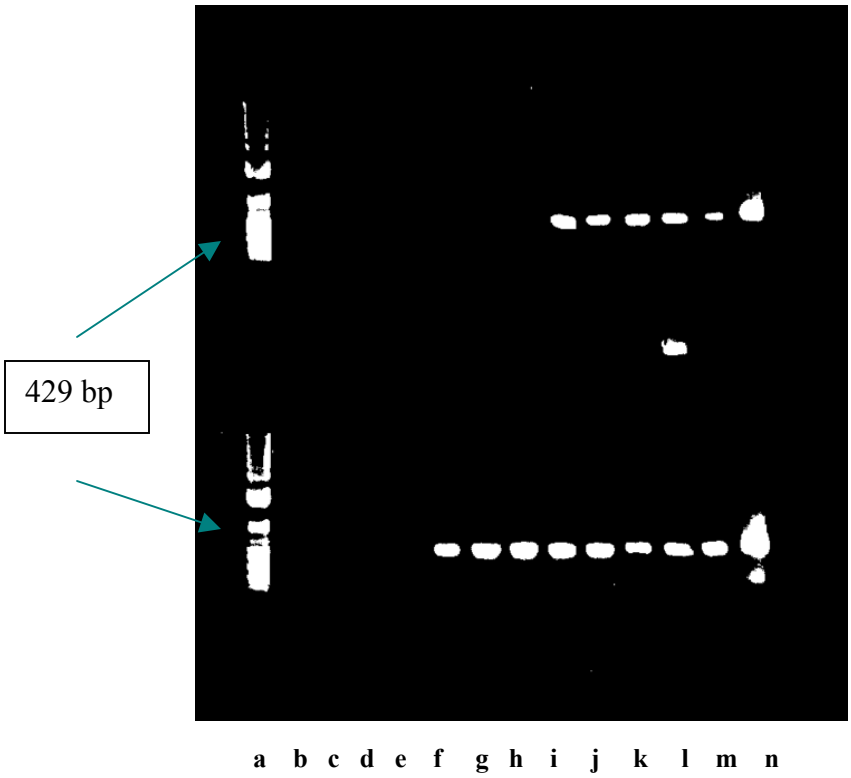


Figure 7: Detection of *Salmonella* strains using PCR primers ST 11 and ST 15. Lane a: 100 bp ladder. Lane b: negative control. Lane c through m: PCR products from naturally contaminated poultry meat samples. Lane n: positive control.



6. Chapter V: Prevalence and Risk Factors for *Salmonella* Contamination of Retail Poultry Meat in Maryland

6.1 Abstract

According to the World Health Organization, *Salmonella* is a leading cause of foodborne illness worldwide. Since the implementation of the HACCP system for pathogen reduction in poultry processing plants, the Food Safety Inspection Service (FSIS) of the US Department of Agriculture reports substantial reductions in the prevalence of *Salmonella* contamination of raw poultry at processing plants. However, limited data is available regarding the prevalence and distribution of *Salmonella* contamination in poultry meat at retail grocery stores. Random samples of retail grocery stores within the state of Maryland were selected for this cross-sectional study. The objectives of this study were to estimate the *Salmonella* prevalence in chicken meat products from retail grocery stores throughout Maryland and to identify risk factors for *Salmonella* contamination in retail poultry meat products. Out of 180 poultry meat samples that were collected, the overall prevalence of *Salmonella* contamination was 22.7% (C.I 15.16-30.86). Integrator brand ground poultry (19%) was at increased risk for *Salmonella* contamination when compared to integrator brand non-ground poultry products (7%) but this difference was not significant (p=0.053). Among non-ground products, store brand poultry was 18 times more likely to be contaminated with *Salmonella* than integrator brand poultry products (CI 5.1-61.2).

6.2 Introduction

Salmonella species are one of the most important foodborne pathogens worldwide. Poultry products have been identified as the primary source for *Salmonella* – associated foodborne outbreaks. According to the Centers for Disease Control and Prevention, *Salmonella* affects about 1.4 million people each year in the United States with about 20,000 hospitalizations and 500 deaths annually.[115]

The Food Safety Inspection Service (FSIS) of the U.S. Department of Agriculture conducts annual surveillance for the prevalence of bacterial contamination of poultry products at the processing plants. Routine surveillance at retail grocery outlets however, is not conducted by FSIS. Inspection and testing of raw meat products at retail outlets falls under the jurisdiction of local departments of health. FSIS is not routinely involved in the testing of raw meat products from grocery stores unless an outbreak is being investigated (personal communication with FSIS personnel).

Prior to the implementation of the Hazard Analysis and Critical Control Point (HACCP) system in poultry processing plants, FSIS nationwide microbial baseline studies reported that at processing plants, *Salmonella* prevalence in whole-raw broilers was 20%, and in ground chicken, 44.6%.[154,156] Since the introduction of the HACCP system in poultry processing plants, substantial reductions in the prevalence of *Salmonella* in raw meat and poultry products were reported by FSIS.[156] FSIS reports post-HACCP *Salmonella* prevalence of 11.4% for whole-raw broilers and 16.2% for ground chicken.[134]

There is limited information in the current literature on the prevalence and distribution of *Salmonella* contamination of raw poultry meat products obtained from

retail grocery stores. A recent study of retail poultry meat products in Spain reported that 35.5% of the products were contaminated with *Salmonella.*[49] *S. enteritidis* was the predominant species isolated[3]. This is consistent with a report from Belgium indicating that 36% of retail poultry meat samples were contaminated with *Salmonella.*[159,160] A recent study in England found that 25% of the retail chicken samples were *Salmonella* positive.[91] In the US, estimates of retail poultry meat *Salmonella* contamination have varied widely. In a 1990 study, Bokanyi and colleagues reported a prevalence of 43% *Salmonella* contamination for retail poultry carcasses and parts.[18] A more recent estimate of retail poultry *Salmonella* contamination in the Washington DC area found 4.2% of carcasses were *Salmonella* positive.[177]

The prevalence of *Salmonella* contamination in poultry meat obtained at retail grocery stores is a better indicator of the public health risk than the processing plant prevalence. It is at the point of the retail grocery store that the consumer typically first comes into contact with the poultry meat. Retail meat may serve as a source of direct hand-to-mouth exposure and cross-contamination exposure to pathogens. Adequate sanitation and chilling may result in an actual decline in the level of viable *Salmonella* isolates in poultry products.[88] However, transportation, handling, storage, additional processing and re-packaging of raw poultry meat products, often occur after the products leave the processing plant. Each of these steps may provide a new opportunity for bacterial contamination or growth. [29]

The objectives of this study were to:

- Estimate the *Salmonella* prevalence in chicken meat products from retail grocery stores throughout Maryland.

- Identify risk factors for *Salmonella* contamination in retail poultry meat products.

Hypotheses:

1. There is no difference in the risk of *Salmonella* contamination of store brand poultry meat products and integrator brand poultry meat products.
2. Ground chicken meat products have a higher risk of *Salmonella* contamination than non-ground chicken meat products.

6.3 Materials and Methods:

Sample Collection

A random sample of 10 retail grocery outlets, representing 10 of Maryland's 22 counties and 4 retail chains were included in the cross-sectional study (Figure 7). Two samples each of: ground chicken, boneless skinless breast filets and bone-in chicken breast portion with skin were collected from each store. A total of 90 raw meat samples were purchased over 8-week sampling intervals in 2003-2004. To increase the probability of detecting *Salmonella*-positive samples, two sub-samples were collected from each of the 90 initial samples obtained. Samples were maintained at 4°C while being transported to the laboratory and then processed within 12 hours of collection.

Pretreatment of sample:

From each sample, two- 25g sub-samples were aseptically collected. Sub-samples were pre-enriched at 37°C in 225 ml of 2% buffered peptone water (BPW) (Becton Dickinson, MD, USA) in a shaking incubator for two hours and placed in 37°C incubator overnight. Ground meat was weighed and placed directly in BPW. Non-ground samples

were weighed, ground, and placed into BPW. A 0.1ml of sample of this stock was transferred to 10 ml of Rappaport Vassiliadis (RV) medium (Becton Dickinson) and another 1 ml of stock was transferred to 10 ml of Tetrathionate Hajna (TT-H) broth (Becton Dickinson). The RV media and TT-H broth were then incubated at 41.5°C for 24 hours. One loopful RV media was streaked onto Xylose Lysine Tergitol (XLT-4) (Becton Dickinson) agar and the plates were incubated at 37°C for 24 hours.[146,170] The same procedure was repeated with samples from the TT-H broth. Presumptively positive (black colony on XLT-4 agar) colonies were bio-chemically confirmed with Triple Sugar Iron (TSI) agar and Lysine Iron Agar (LIA) tests, and also, confirmed by serum "O" and "H" agglutinating antisera (Murex Diagnostics, Kent, UK; Behring, Germany) testing.

Data Analysis:

Data was stored in Microsoft Excel spreadsheets and descriptive statistics were calculated. The Chi-square test[39] was used to test the stated hypotheses using analytical software – (Statistix-8, Tallahassee, FL). To identify risk factors for *Salmonella* contamination of the meat samples, four categorical independent variables were presented to the logistic regression model[39]: processing of meat (ground, non-ground), presence of skin (with skin, skinless), retail outlet (outlet names) and product brands (store-brand, integrator brand).

6.4 Results:

The results of the univariate analyses are summarized in Tables 13,14 and 15. Overall the prevalence of *Salmonella* contamination in poultry meat products was 23%

(C.I 15.16 - 30.86). Ground chicken meat did not have a significantly increased *Salmonella* prevalence when compared to non-ground breast meat products. Integrator brand samples represented 80% (144/180) of the products sampled while 20 %(36/180) were store brands. None of the store brands represented ground chicken meat because store-brand ground meat products were not available at any of the retail outlets sampled. Store brands had a significantly higher prevalence of *Salmonella* positive samples than did integrator brand products. In non- ground meat category, store brand meat products were 18 times more likely to be contaminated with *Salmonella* than integrator brand (C.I. 5.41-61.26).

6.5 Discussion:

This study found an overall *Salmonella* prevalence of 23% for poultry meat products at the retail grocery store outlets. This level of contamination is consistent with the recent literature from European countries such as England (25%), Spain (35%), and Belgium (36%) but much lower than a 1990 USDA estimate of 43%. Interestingly, the level of contamination found at retail grocery stores is similar to the national baseline pre-HACCP levels reported by FSIS (20% for carcasses and 44.5% for ground meat).[18,49,154,156,159] After implementation of the HACCP guidelines, FSIS reported a decline in *Salmonella* prevalence to 11% for carcasses and 16% for ground chicken at US processing plants.[134]

The findings from this study are of significance to public health because they indicate that the potential benefit of the post-HACCP reduction in *Salmonella* prevalence at the processing plant is being undermined by contamination occurring at the retail

grocery store outlet. This conclusion is supported by the findings from the ground chicken products. No store brand ground chicken products were available from any of the retail outlets sampled in this study. The *Salmonella* prevalence from the integrator brand ground chicken products in this study (19%) is similar to the FSIS post-HACCP *Salmonella* prevalence for ground chicken obtained at the processing plant (16%).

Not only is there a limited quantity of information in the literature regarding the prevalence of *Salmonella* in poultry products sampled from retail grocery store outlets, but a considerable variation in those reports as well. Contrary to the previously cited studies from the US and Europe with retail poultry products *Salmonella* prevalence estimates ranging from 25-43%, a recent study in the Washington DC area reports a *Salmonella* prevalence of only 4% from retail poultry carcasses.[177] Several factors may have contributed to these very different estimates of *Salmonella* prevalence. The methods used for obtaining the samples from the poultry products were not consistent across the different studies. The potential impact of the differences in methodology has been documented in the literature. Uyttendaele and colleagues report a 100% increase in the *Salmonella*-positive samples when carcasses were cut into individual parts before sampling rather than using whole carcass-rinse samples.[159] Jorgensen and colleagues report that *Salmonella* was more frequently isolated from samples containing chicken skin in comparison with those containing carcass-rinse fluid only.[91] Differences between studies in the media used for enrichment, selective enrichment, and isolation can also affect estimates of prevalence.[23,42,43]

The limited literature available on the prevalence of *Salmonella* contamination of poultry products at the retail grocery outlet reveals an important deficiency in the body of

knowledge on the epidemiology of human salmonellosis. An accurate assessment of the risk of human foodborne salmonellosis requires this vital piece of information. The probability of *Salmonella* contamination of a poultry product obtained at the retail grocery outlet differs from estimates based on processing plant *Salmonella* contamination prevalence. Integrator brand products have been packaged at the processing plant. However, conditions during transportation and holding at the retail outlet, integrity of the packaging materials, and opportunities for cross-contamination can result in large changes in the risk of salmonellosis to the consuming public.

Store brand products are subject to the same potentials for additional contamination as integrator brand products. Perhaps of even greater potential risk is that store brand products are often re-packaged, handled, or further processed at the retail grocery store outlet prior to sale to the consumer. The lack of uniform surveillance protocols for raw poultry products at the retail grocery outlet constitute an important gap in the farm-to-fork spectrum of food safety protection and a risk to public health and food safety that needs to be addressed.

Figure 7: Sampling sites for 2003-2004 cross-sectional study of the prevalence of *Salmonella* contamination in retail chicken met products in Maryland. N = 180

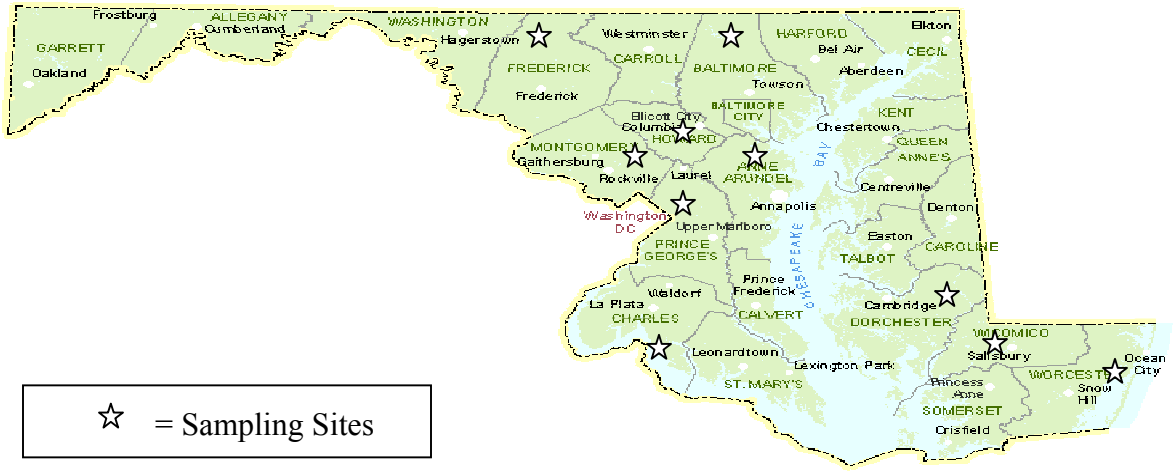


Table 13. Univariate analysis of ground chicken versus non-ground chicken (breast meat) products as a risk factor *Salmonella* contamination of retail chicken meat products in Maryland, sampled in 2003-2004. (N=180; 42 positive samples).

	N	Number *Salmonella* Positive	Percent *Salmonella* Positive	P-value (Chi-square)
Ground-Chicken	88	17	19	0.2129
Non-Ground Chicken	92	25	27	
Total	180	42	22.78	

Table 14. Univariate analysis of integrator brand products, ground chicken versus non-ground chicken (breast meat) as a risk factor *Salmonella* contamination of retail chicken meat products in Maryland, sampled in 2003-2004. (N=144; 21 positive samples).

Integrator Brand	N	Number *Salmonella* Positive	Percent *Salmonella* Positive	P-value
Ground	88	17	19.32	0.0758
Non-ground	56	4	7.14	
Total	144	21	14.58	

Difference = 0.1218, 95% C.I of difference 0.0035 – 0.2400

Table 15. Univariate analysis of non-ground chicken products*; brand-type as a risk factor for *Salmonella* contamination of retail chicken meat products in Maryland, sampled in 2003-2004.

	N	Number *Salmonella* Positive	Percent *Salmonella* Positive	P-value
Store Brand*	36	21	58.33	**<0.0001♣**
Integrator Brand	56	4	7.14	
Total	92	25	27.17	

* No store brand ground chicken products were available for purchase at any of the retail outlets sampled. Only boneless-skinless breast filets and bone-in breast meat with skin were available as store-brand items.

♣ Statistically significant at (α =0.05)

7. Summary and Conclusions

The 2001 Food Safety Strategic Plan of the President's Council on Food Safety calls for safety control efforts at every stage "from farm to fork" including enhancement of national, systematic monitoring of food animal diseases and testing of feeds and feedstuffs for microbial, chemical, and other hazards that pose a food safety risk. Control of *Salmonella* has been a big challenge not only for the poultry industry but also for public health officials. This dissertation describes studies conducted regarding the epidemiology of *Salmonella*. A review of the literature identified four subject areas where there was insufficient data on the epidemiology of *Salmonella*. These so-called gaps in the body of knowledge are distributed throughout the farm-to-fork model of poultry production including risk factors in the on-farm environment, diagnostic tools for surveillance, and risk factors for product contamination at the point of retail distribution. The results of our research have highlighted the challenges facing those seeking to control *Salmonella*. Each of these studies has been preliminary in nature and due to their cross-sectional design, their ability to clearly demonstrate cause and effect relationships is limited. However, in spite of these constraints, several important conclusions may be drawn from these findings.

Although *Salmonella* contamination of the plant-based feed samples evaluated in this study was low, it still demonstrated that animal feed is contaminated with enteric bacterial pathogens and that seasonal patterns of contamination may be an important risk factor to consider when designing feed monitoring programs. Microbial contamination in animal feed should be a great concern and improvement in the safety of animal feed to reach the 1991 FDA stated goal of *Salmonella*-free feed would require two major

measures. First, surveillance of animal feed for microbial contamination is necessary, which must be integrated with surveillance systems used in food animals, food, and humans. Second, a HACCP program is needed in the animal feed industry to minimize bacterial contamination by identifying and controlling sources of feed contamination.

Relative humidity and airflow over the litter surface represents potentially important poultry house parameters that need to be monitored to ensure a safe and healthy environment for broiler performance by taking appropriate measures, which could involve improving the uniformity of moderate airflow rates of at least 1.5 mph in lower humidity regions and at least 3.5 mph in higher humidity regions.

In order to compare findings across geographic regions and evaluate protocols, the techniques for *Salmonella* detection need to be standardized. When using a technique that includes both pre-enrichment for 12 hours and selective enrichment, PCR has proven to be as sensitive as bacterial culture method while providing results within 16-24 hours.

Lastly, the *Salmonella* prevalence of 23% reported in this study for poultry meat products at the retail grocery store outlets is similar to the national baseline pre-HACCP level reported by FSIS. Since the post-HACCP level of *Salmonella* contamination reported at processing plants is substantially lower, this indicates that the potential benefit of the post-HACCP reduction in *Salmonella* prevalence at the processing plant is being undermined by contamination occurring after distribution from the processing plant, perhaps at the retail grocery store outlet. The lack of uniform surveillance protocols for raw poultry products at the retail grocery outlet constitutes an important gap in the farm-to-fork spectrum of food safety protection and a risk to public health and food safety that needs to be addressed.

Extensive research on the epidemiology of *Salmonella* in poultry products, and its role in human salmonellosis, has been conducted. However, contamination of products persists and may be growing. While this set of research projects has helped to close some of the gaps in the body of knowledge on the epidemiology of *Salmonella* in poultry and poultry products, it has also identified areas where additional research needs to be conducted. Further prospective studies are still needed to identify risk factors for bacterial contamination of animal feed and retail meat; a retail outlet HACCP program for meat and poultry products needs to be developed and evaluated for efficacy, and more extensive studies on airflow and perhaps the use of ceiling fans to correct the stagnant airflow spaces, are needed to verify and validate effective control and prevention of microbial contamination.

A science-based approach to food safety issues is data intensive. Data is needed for risk assessment, identification of points of entry into the food chain, tracking substances as they move through the food system, analyzing the behavior and perceptions of market participants, communicating disparities between real and perceived risks, and measuring the costs and benefits of alternative control points. In addition, the changing nature of scientific knowledge in the food safety area means that new possibilities for assessing and controlling food safety risk are emerging. Current progress on identifying microbial risks and sources in food is pointing the way to control options. However, additional data are needed to conduct benefit/cost analyses of alternative control options, which will require interdisciplinary cooperation to combine the underlying physical and technical relationships with the economic and behavioral aspects of market participants.

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