The risk of *Salmonella* spp. from small poultry operations in Oregon

by
Jonathan Diaz-Hui

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented May 23, 2016
Commencement June 2016

AN ABSTRACT OF THE THESIS OF

Jonathan Diaz-Hui for the degree of Honors Baccalaureate of Science in Microbiology presented on May 23, 2016. Title: The risk of *Salmonella* spp. from small poultry operations in Oregon.

Abstract approved:

_____

Joy Waite-Cusic

Salmonella is a bacterium responsible for several illnesses, including typhoid fever, paratyphoid fever, and salmonellosis. It is commonly found in animal products, including poultry. Determination of potential reservoirs of *Salmonella* contamination is important to mitigate the risk of transmission and possible outbreaks. Outbreaks can negatively impact the livelihood of local Oregon farmers exempt from continuous federal inspection. The objective of this study was to determine potential reservoirs for *Salmonella* contamination at an Oregon poultry operation. Results indicated that chicken litter and water fountains were the reservoirs for contamination. Future studies could identify mitigation strategies that prevent *Salmonella* transmission through treatments targeting these reservoirs.

Key Words: *Salmonella,* poultry

Corresponding e-mail address: diazhuij@oregonstate.edu

The risk of *Salmonella* spp. from small poultry operations in Oregon

by
Jonathan Diaz-Hui

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented May 23, 2016
Commencement June 2016

Honors Baccalaureate of Science in Microbiology project of Jonathan Diaz-Hui presented on May 23, 2016.

APPROVED:

Joy Waite-Cusic, Mentor, representing Department of Food Science and Technology

Walt Ream, Committee Member, representing Department of Microbiology

Theo Dreher, Committee Member, representing Department of Microbiology

Toni Doolen, Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Jonathan Diaz-Hui, Author

Table of contents

Table of contents 1

Literature review 3
- **Poultry production and consumption in the United States** 3
- **Federal regulation of poultry products and exemptions** 3
- **Oregon poultry exemptions** 4
- **Recent *Salmonella* outbreaks associated with poultry** 5
- **Rates of *Salmonella* spp. contamination in poultry** 5
  - Small-scale poultry production and rates of contamination 6
- **Overview of Salmonella spp.** 6
  - *Salmonella* spp. nomenclature 7
  - *Salmonella* and Salmonellosis 8
- **The USDA Microbiology Laboratory Guidebook (MLG).** 8
- **Selective enrichments** 9
  - Rapport-Vassiliadis (RV) broth. 9
  - Tetrathionate (TT) broth. 9
- **Isolation Medium** 10
  - Do all *Salmonella* produce black colonies on all media? 12
- **Secondary identification step** 12

Approach and Objectives of the Current Study 13

Materials and methods: 14
- **Poultry farm** 14
- **Sample Collection - Water** 15
- **Sample Collection – Litter and Feed** 15
- **Sample Collection - Chicken** 16
- **Sample Analysis – *Salmonella* spp.** 16

Results: 17
- ***Overall Salmonella prevalence*** 17
  - *Figure 1. Percentage of Salmonella positive isolates.* 19
- **Positive sample analysis** 20
  - Figure 2. Salmonella prevalence amongst individual bird ages/broods 20
- **Polymerase chain reaction lack of results** 22
- ***Salmonella* serotyping** 23

Discussion 23
- **Individual brood prevalence** 23
- **Salmonella serotyping** 24
- **Control strategies - Water** 24
- **Control strategies - Litter** 25

Reference: **26**

Appendix 1 ..... **28**

Photo 1. Chick (about 1 day in age) **28**

Photo 2. Chicks with a feeder for their specific age **29**

Photo 3. Example of water fountain **29**

Photo 4 (1-2 weeks in age) **30**

Photo 5. Chicken age 3-4 weeks **30**

Photo 6. Chickens age 7 weeks + ... **31**

Literature review

Poultry production and consumption in the United States

Poultry is one of the main meat products consumed in the United States, with the availability of chicken meat doubling since 1970 (1). According to the North American Meat Institute, American meat companies produced 38.4 billion pounds of chicken in 2013, making it the highest produced meat product that year (2). It overtook pork as the second most consumed meat in 1996 (1), with a 92.1 pounds per capita consumption of chicken estimated in 2016 (3).

Federal regulation of poultry products and exemptions

Chicken products in the United States are subject to regulations under the Poultry Products Inspection Act of 1957. This legislation requires that the USDA inspect domestic birds (such as turkeys and chickens) when slaughtered and during processing. This is to ensure that all products are unadulterated and safe for human consumption when it is sold between states or imported (4); however, poultry operations may be exempted from continuous USDA inspection if a series of criteria are met (Table 1).

Table 1. Exemption criteria for continuous USDA inspection of poultry processors. (5).

- Legal reference: PPIA Section 464(c)(1)(C) &(c)(3) "Section 15 (c)(4)" and Title 9 CFR §381.10(a)(5) and (b)(1) and (2).

Exemption	Criteria
1	The producer/grower slaughters and processes, on his or her own premises, no more than 20,000 poultry, raised by him or her, in a calendar year.
2	The producer/grower sells, in a calendar year, only poultry or poultry products he or she prepares according to the criteria for the Producer/Grower – 20,000 Limit Exemption; he or she may not buy or sell poultry products prepared under another exemption in the same calendar year in which he or she claims the Producer/Grower – 20,000 Limit Exemption.
3	The poultry products are distributed solely by the producer/grower and only within the District of Columbia or the State or Territory in which the poultry product is produced.
4	The poultry are healthy when slaughtered.
5	The slaughter and processing at the producer/grower's premises are conducted using sanitary standards, practices, and procedures that produce poultry products that are sound, clean, and fit for use as human food (not adulterated)
6	The producer only distributes poultry products he or she produced under the Producer/Grower Exemption
7	The facility used to slaughter or process the poultry is not used to slaughter or process another person's poultry unless the Administrator of FSIS grants an exemption [PPIA Section 464(c)(3); Title 9 CFR 381.10b)(2)]
8	The shipping containers, when distributed in intrastate commerce (instead of the required features of a label of inspected product) bear: the producer's name, producer's address, and the statement "Exempt P.L. 90-492."

Oregon poultry exemptions

In 2011, the Oregon State Legislature passed House Bill 2872. This bill gave small poultry operations more options for processing and selling their product (6). In Oregon, there are 20 state-licensed poultry operations that produce less than 20,000 birds/year (the federal exemption) that are exempt from continuous federal inspections by the USDA as per the Poultry Products Inspection Act (5,7). Certain criteria must be met for exemption, including following established federal, state, and local law. During the calendar year the facility can slaughter a maximum 1,000 poultry, ensuring they are free of disease and used for human food (8). Sanitation standards established in the Oregon Department of Agriculture ORS 619.026 must be followed such that all equipment "shall be kept in a clean, healthful and sanitary condition" (6). In addition, sanitary records under OAR 603-

028-0740 must be kept to indicate factors such as date of poultry slaughter, cleaning records, and species and quantity of poultry sold (8).

Recent *Salmonella* outbreaks associated with poultry

In 2013, there was an outbreak of multi-drug resistant *Salmonella* Heidelberg infections linked to Foster Farms brand chicken after 634 people consumed infected meat (9). The outbreak occurred in 29 different states and Puerto Rico, with 38% of people being hospitalized and 77% of instances being from California. The resistance to several common antibiotics for this particular strain could increase the risk of hospitalization. In September 2013, USDA Food Safety and Inspection Service (FSIS) members conducted tests to identify *Salmonella* spp. at four establishments in California and Washington, later finding 6 out of 7 outbreak strains within California facilities. These discoveries ultimately led Foster Farms to recall an undetermined amount of chicken product in July 2014. Those products were likely to be contaminated with *Salmonella*. Control measures by Foster Farms greatly reduced *Salmonella* prevalence, with the FSIS determining that the firm's control measures were successful since the recall. (9).

Rates of *Salmonella* spp. contamination in poultry

The FSIS sets specific standards for the number of positive *Salmonella* samples from poultry products, including ground chicken, ground turkey, and chicken parts (10). The 2015 changes were made to address poultry products that are more common than whole birds, which was the performance baseline in 1996. New standards allow a maximum

25% *Salmonella*-positive in ground chicken and 15.4% *Salmonella*-positive in chicken parts, significantly lower than the old standard for ground chicken at 44.6% (10). If this performance standard weren't met, the FSIS would conduct tests to ensure that the establishment is giving its best effort to improve food safety. This effort was part of the 2013 Salmonella Action Plan to reduce illness caused by *Salmonella* spp. Poultry accounted for 58% of all Salmonellosis cases in FSIS-regulated products, with 85% of this amount associated with chicken and 15% associated with turkey (10).

Small-scale poultry production and rates of contamination

The increase in demand for local foods at farmers markets has given many farmers a chance to expand their businesses without continuous inspection by federal agencies thanks to meeting exemption criteria under the Poultry Products Inspection Act (4, 11). Researchers at Pennsylvania State University analyzed 100 whole chickens from farmers markets, 50 conventionally processed whole chickens, and 50 USDA certified organic whole chickens. This was to determine the risk of harboring pathogens for each category (11). Results showed that farmers market chickens had a higher contamination percentage compared to the organic and conventional methods; 28% of whole farmers market chickens were positive compared to 20% in conventional methods and 5% under USDA certified organic methods (11).

Overview of Salmonella spp.

Salmonella are Gram-negative bacteria that are responsible for foodborne illness, with *Salmonella enterica* subsp. *enterica* serovars Enteritidis and Typhimurium being the most common serotypes in the United States (12). Commonly ingested with contaminated food or water, *Salmonella* spp. is frequently found in food derived from animals such as beef, eggs, and poultry. This bacterium is a serious public health concern; the Centers for Disease Control and Prevention estimates that 1.2 million illnesses and 450 deaths from *Salmonella* infection occur annually in the United States (13).

Salmonella spp. nomenclature

Salmonella possess three specific antigens, namely H, O, and Vi (14). H antigen (the flagellar antigen) occurs as either phase 1 or 2, with possible changes in phase. O antigen is present on the outer membrane and based on unique sugar sequences (14). Vi is an addition to the O antigen that is present in a few serotypes like *S.* Typhimurium (15). The three Salmonella antigens determine serotypes, classified under the Kaufmann-White scheme (15). After listing the serotype further categorization is as follows (15):

- O antigens (including Vi) are listed first followed by H antigens.
- Colons separate major antigens and commas separate the components of the antigen.
- Underlined O indicates it is encoded by a bacteriophage.
- Square brackets [] indicate the factor may not be present or encoded by a bacteriophage.
- Curly brackets {} indicate the factor is exclusive.
- Parenthesis () indicate the factor is weakly joined.

Salmonella and Salmonellosis

Salmonellosis is the disease resulting from infection by *Salmonella* bacteria. Symptoms include abdominal cramps, diarrhea, and fever after consuming contaminated foods. The symptoms disappear after a few weeks; however, infections are life threatening for young children, the elderly, and the immunocompromised (13).

The USDA Microbiology Laboratory Guidebook (MLG).

The MLG is a guidebook with protocols and tests used during FSIS inspections of meat, poultry, and egg products. The MLG protocols specifically detail sample preparation, isolation, and identification of foodborne pathogens and their toxins (16). These standardized procedures were used for all media preparation in this study. There were differences in the methodology between this study and MLG Appendix 2.02 specific for laboratory analysis of *Salmonella*:

- Triple iron sugar (TSI) or Lysine iron agar slants were not used following the selective media isolation.
- Instead of using sheep blood agar to streak for purity, presumptive *Salmonella* colonies found on each differential agar medium was transferred on the proprietary CHROMagar *Salmonella* Plus medium.

Several different selective growth media were used throughout this study. Various broths and agars were used to provide an identification of *Salmonella*-positive isolates.

Selective enrichments

Two different *Salmonella*-selective enrichments were used in the study, Rapport-Vassiliadis and Tetrathionate broths. Both are used for FSIS analysis outlined in the MLG, which is why the two were used. From the results of the March 3, 2015 isolates it did not appear that either enrichment was more efficient than the other. Afterwards only Tetrathionate was used for the remainder of the study since this was the most readily available medium.

Rapport-Vassiliadis (RV) broth.

This broth medium is used to select for *Salmonella* spp. due to the components of the broth. Malachite green and magnesium chloride are used to select for bacteria like *E. coli* or *Salmonella* (17). Malachite green inhibits organisms other than *Salmonella* spp. and *E. coli* and magnesium chloride raises the osmotic pressure of the medium (17). The presence of these two compounds creates a selective medium for *Salmonella*. Soy-peptone is the carbon and nitrogen source of this medium (17).

Tetrathionate (TT) broth.

This broth medium is used for selective enrichment of *Salmonella* spp. when bacteria compete with intestinal flora. Its high selectivity makes it a standard for *Salmonella* testing procedures (18). Enzymes of the bacteria digest casein to provide all needed nitrogen, carbon, and amino acids. Upon addition of iodine and potassium iodide to the

solution, only organisms with tetrathionate reductase can proliferate successfully. The bile salts present in the media inhibit growth of Gram-positive organisms by dissolving their plasma membranes (19).

Isolation Medium

All isolation media were prepared following the USDA MLG protocol. Multiple media were used to provide better isolation of individual *Salmonella* colonies. This was to provide some assurance in case a colony on one medium was not *Salmonella*-typical while the other was *Salmonella*-typical. The MLG includes other media besides the two used in this study, such as Triple sugar iron agar (TSI), lysine iron agar (LIA), and Brilliant green sulfa agar (BGS) (16). While all of these media are selective for *Salmonella* based on biochemical tests targeting *Salmonella* spp., the MLG notes that media selection can be augmented for epidemiological purposes (16). The Hektoen Enteric and Xylose-Lysine-Deoxycholate agars were used in this study, following standard USDA formulation.

Hektoen Enteric agar (HE agar). This agar is selective for Gram-negative organisms. Like TT broth, HE agar contains bile salts to inhibit the growth of Gram-positive organisms. Several fermentable substrates within the medium (including lactose, sucrose, and salicin) in combination with pH indicators (bromthymol blue and acid fuchsin) assist in colony differentiation (20). *Salmonella* do not ferment these substrates, leaving the agar dark green (20). In contrast, *E. coli* and many other enteric bacteria do ferment these substrates into acidic end products and cause the medium to turn bright yellow. *Salmonella*-typical colonies are differentiated by green to blue-green colonies with a

black center. *E. coli* colonies lack the black center of *Salmonella* spp. Instead they appear yellow with orange halos (20). Colonies turn black because of ferric ammonium citrate and sodium thiosulfate in the agar (20). *Salmonella* spp. contain enzymes that release sulfide from the medium and the sulfide is coupled with hydrogen to form hydrogen sulfide gas (21). By reacting with ferric ammonium citrate, the hydrogen sulfide gas forms a precipitate causing black colonies distinct for this medium (21). Nonpathogenic bacteria such as *Proteus* and *Citrobacter* spp. can produce hydrogen sulfide gas but they are inhibited by bile salts in the HE agar.

Xylose-Lysine-Deoxycholate agar (XLD agar). This agar is used to select lactose fermenters and differentiate based on hydrogen sulfide. Sodium deoxycholate inhibits the growth of Gram-positive bacteria as a detergent that disrupts their cell membranes (22). Lysine is used to differentiate *Salmonella* spp. from non-pathogens since they would normally ferment xylose, and loses any distinguishing *Salmonella* characteristics (23). After xylose is expended, lysine decarboxylase attacks available lysine and generates an alkaline pH. Further differentiation is based on *Salmonella* having unique black centers to their colonies. This indicates the formation of hydrogen sulfide using sodium thiosulfate and ferric ammonium citrate (23), as in HE agar. Non-pathogenic hydrogen sulfide producers do not decarboxylate lysine. No black precipitate is produced in this case because the reaction only occurs at neutral or alkaline pH (23).

Do all *Salmonella* produce black colonies on all media?

In this study, *Salmonella* typical colonies usually appeared black due to hydrogen sulfide formation. This was the case in the HE and XLD agar mentioned previously. However, some serotypes of *Salmonella* spp. do not readily produce hydrogen sulfide. In the case of the media used in this study, hydrogen sulfide negative *Salmonella* would produce colonies that match the media color instead of black colonies (23). Also, hydrogen sulfide production is distinct to certain serotypes, namely *Salmonella* Typhimurium. Hydrogen sulfide production is less common in serotypes like Derby and Heidelberg (24). Previous studies show that these serotypes can be a public health concern and are found with common serotypes like Enteritidis and Typhimurium. Besides serotype differences, colony morphology can differ based in the selective media.

Secondary identification step

Following differential and selective isolation, the MLG procedure uses further conformational tests with tryptic soy agar with 5% sheep blood (sheep blood agar, SBA). The plates are normally streaked following positive biochemical tests from triple sugar iron (TSI) and lysine iron agar (LIA) slants (16). Instead of using SBA, this study used a proprietary medium called CHROMagar *Salmonella* plus. This agar was used because the colonies vary in color (pink, blue, or green) and are clearly distinguishable over the colorless medium.

CHROMagar. This agar is a unique formulation developed by Dr. Alain Rambach in 1989 (25). The chromogenic agar is used to detect *Salmonella* spp. pathogens. The mechanism of action is through a soluble colorless molecule consisting of a substrate and a

chromophore (25). The chromophore targets a specific enzymatic activity within the target organism. Once the enzyme cleaves the chromogen, the chromophore precipitates and generates a distinct color (25). Since this is a proprietary media, details on the specific enzyme and chromophore are not readily available. Mauve to purple coloration indicates an identification of *Salmonella* spp. Blue, white, or colorless colonies are typically *Proteus* spp., *E. coli*, or coliforms.

Approach and Objectives of the Current Study

Previous work in the Waite-Cusic laboratory identified a small-scale poultry processor in Oregon that had a high prevalence of *Salmonella* spp. in dressed poultry carcasses. With a higher prevalence of positive samples, this farm is at risk of being associated with an outbreak. Environmental sampling in the processing facility determined that the production operation was contributing to a large number of live birds carrying *Salmonella*. The purpose of the current study was to explore the farm environment for a potential reservoir. The identification of a reservoir could lead to an approach to effectively reduce the spread of *Salmonella* on-farm, which would reduce the presence of *Salmonella* in the processing environment. Effective implementation of these changes could lead to safer food products and a sustainable livelihood for small farm operations.

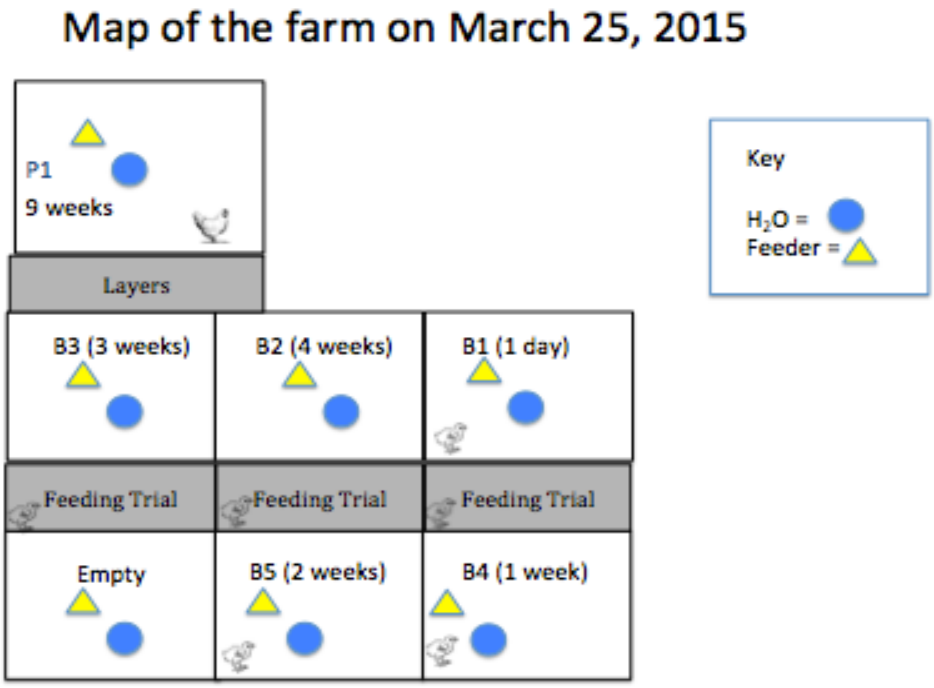
Materials and methods:

Poultry farm

Samples were collected from a local farm previously associated with high rates of positive *Salmonella* isolation in dressed poultry carcasses. Samples of water, litter, feed, and birds were collected between March and August 2015. Chickens broods were categorized by age and sampled in order of increasing age. Individually prepared bags containing all sample-gathering materials were used to minimize cross-contamination between areas of the operation.

Individual broods and pens were categorized and mapped in the following manner:

Layout 1 and 2: Maps of the poultry farm on different dates.



Map of the farm on May 21 and August 11, 2015

Sample Collection - Water

Water samples (50 mL) were gathered in sterile 50-mL conical vials using an automatic pipette. Samples were gathered from all water dispensers. Samples were transported back to the laboratory and each water sample (0.2, 1, 2 mL) was transferred to buffered peptone water (BPW; 1:10) and incubated at 37°C for 24 hours. This was made using USDA guidelines and formulation.

Sample Collection – Litter and Feed

Litter and feed samples were collected from various brood pens throughout the poultry housing facility. Clean litter and feed samples were also collected from the original packaging. Litter samples (~ 100 g) were collected in sterile Whirl-Pak bags (Nasco) and feed samples were collected in sterile 50-mL conical tubes. Samples were transported back to the laboratory and subsampled (10 g) for enrichment in buffered peptone water

(BPW) or lactose broth (1:10) and incubated at 37°C for 24 hrs. These enrichments were made using USDA guidelines and formulation.

Sample Collection - Chicken

Random birds from each brood (n = between 24 and 60 birds) were captured and rectally swabbed with 3M brand Quick swabs. Swabs were immersed in buffered peptone water (BPW) and transported to the laboratory. Swab tip and suspension media were transferred to BPW (10 mL) and incubated at 37°C for 24 hrs.

Sample Analysis – *Salmonella* spp.

Following incubation of primary enrichment (BPW or Lactose Broth), samples (0.1-0.5 mL) were transferred to selective secondary enrichment media (10 mL): Tetrathionate Broth (TT; Hajna formulation) and/or Rappaport-Vassiliadis broth (RV). 0.5 mL of sample was transferred into 10-mL tubes of TT; 0.1 mL of sample was transferred into 10-mL tubes of RV. Selective enrichments were incubated at 37°C for 24 hrs. Following selective enrichment, samples were streaked for isolation on selective-differential media: Hektoen Enteric Agar (HE; Neogen) and Xylose Lysine Deoxycolate Agar (XLD; Neogen). These plates were incubated at 37°C for 48 hrs. Colony morphology was examined, and typical black colonies were transferred to Chrom Agar Salmonella Plus (DRG International) and incubated at 37°C for 24 hrs. Colonies displaying pink-purple color were considered confirmed as *Salmonella*. Confirmed isolates were transferred to

TSB for a final enrichment and incubated at 37°C for 24 hrs prior to storage at -80°C in 30% glycerol.

Results:

Overall Salmonella prevalence

On March 3 2015, 12 of the 76 samples gathered tested positive for *Salmonella* using both the HE/XLD agars and Chrom agar. All 12 positive samples were from the environment where the chickens roosted, indicating that some *Salmonella* contamination was present on the farm. These samples served as a preliminary indicator of whether or not *Salmonella* spp. was still on the farm. 39 other samples showed blue growth on Chrom agar, indicating that the isolated colony was *E. coli* and not *Salmonella.* The remaining 25 samples did not have any growth.

On March 25, 2015, 48 total samples were gathered amongst 6 broods of varying ages. 4 chicken samples, 2 litter samples, 1 water sample, and 1 feed sample were gathered for each group. Photos of the various different age groups and environments are shown in Appendix 1. Ages ranged from 1 day to approximately 9 weeks in age. More time in the environment did not appear to influence *Salmonella* prevalence in individual broods on this date, with the 1-week brood having the highest number of positive samples, 5 out of 8 samples for that brood tested positive for *Salmonella*. In total, the water samples had the highest prevalence of *Salmonella*-positive isolates, with 83.33% (5/6) positive amongst all six groups. The second highest total prevalence was the litter that the birds

roosted in, which had 75% (8/12) *Salmonella*-positive. The third highest total prevalence was in the chicken feed with 33.33% (2/6) samples positive. Individual chickens had the smallest percentage of *Salmonella* positive samples, with 12.50% (3/24) of samples testing positive for *Salmonella* on both the selective media and Chrom agar.

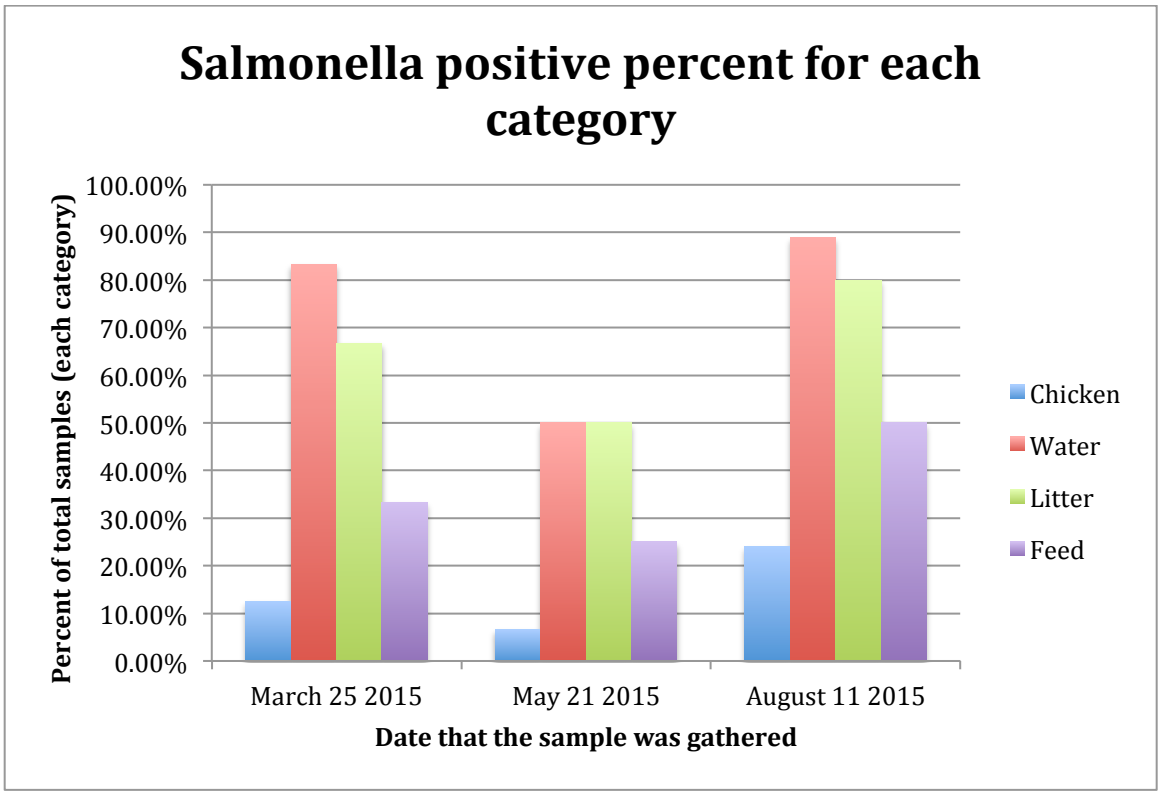
On May 21, 2015, 108 samples were gathered amongst six broods, including previously untested holding pens. Broods and pens each had 10 chicken samples, 2 water samples, 2 litter samples, and 4 feed samples. Maps of the approximate layout of the farm, including revisions to the brood names, are on page 15. Chickens varied from 1 week in age to 9 weeks in age. Brood 5 (age 3 – 4 weeks) had a mixed population, including both chickens and turkeys. Interestingly this brood contained the most samples (10 samples) that were positive on HE/XLD yet negative on Chrom agar. We omitted gathering samples from Brood 4 because the brood was in poor condition and far too sick to allow proper sample gathering. Pens containing broods from 8 – 9 weeks of age had the highest number of *Salmonella*-positive samples, including 100% of the water and litter samples testing positive for *Salmonella.* Results from May 21 were similar to March 25. In this case, 50% of all 12 litter and 12 water samples tested positive for *Salmonella*. This was followed by 25% positive (6/24 total) feed samples and 6.67% positive (4/60 total) chicken samples.

On August 11, 2015, 65 samples were gathered from three different broods. All chickens during this day were roughly the same age at 8 weeks. Much fewer samples were gathered as a result, with 25 chicken samples, 20 litter samples, and 2 feed samples gathered amongst the three broods. Water samples were divided into dilutions as

previously described. Nearly all of the 18 water samples (88.89%) tested positive for *Salmonella*. As in the previous days, litter samples had the second highest percent of positive isolates (80% amongst all three broods) followed by feed samples (50% amongst all three broods) and chicken samples (24% amongst all three broods). Results are also shown in Figure 1.

Figure 1. Percentage of Salmonella positive isolates.

This graph shows the overall percentage of *Salmonella* positive isolates divided by category from each date. Water and litter samples had a consistently higher number of positive samples than individual chickens or chicken feed. This suggests that the water and litter serves as reservoirs for contamination.



Positive sample analysis

Breaking down overall *Salmonella* positive results into individual brood groupings does not show a consistent correlation between older chicken age and number of positive isolates. If a bird spent a longer time period in the same contaminated environment, it would be expected that it would be exposed more frequently and thus have a larger percent positive for a given brood or pen. The March 25, 2015 results seem to contradict this hypothesis because brood 4 (age 1 week) had many more positives than any of the older broods. On March 25 and August 11, 2015 there was a higher prevalence amongst all reservoir types for older broods/pens compared to younger ones. The prevalence breakdown is located in Figure 2.

Figure 2. Salmonella prevalence amongst individual bird ages/broods

The previous figure accounts for the overall prevalence in each category. The following table divides results into each reservoir and age category. If one category did not have positive isolates it was not included.

- March 25: Chicken had 4 samples, litter 2, feed 1, and water 1 for each brood or pen.
- May 21: Chicken 10, litter 2, feed 4, water 2 for each brood or pen.
- August 11: Chicken 25 (Pen 2 only), litter 10, feed 3, and water 6 for each pen. Water samples were split into a two-enumeration scheme replicate using the scheme on page 15.

March 25, 2015

Brood/Pen number	Brood/pen age	Isolate type	Percent positive
Brood 1	1 day	Chicken	25.00%
Brood 1	1 day	Water	100.00%
Brood 2	4 weeks	Litter	50.00%
Brood 2	4 weeks	Water	100.00%
Brood 3	3 weeks	Chicken	25.00%
Brood 3	3 weeks	Litter	100.00%
Brood 3	3 weeks	Feed	100.00%
Brood 4	1 week	Chicken	25.00%
Brood 4	1 week	Litter	100.00%
Brood 4	1 week	Feed	100.00%
Brood 4	1 week	Water	100.00%
Brood 5	2 weeks	Litter	50.00%
Brood 5	2 weeks	Water	100.00%
Pen 1	9 weeks	Litter	100.00%
Pen 1	9 weeks	Water	100.00%

May 21, 2015

Brood/Pen number	Brood/pen age	Isolate type	Percent positive
Brood 1	4 weeks	Water	50.00%
Brood 1	4 weeks	Litter	50.00%
Brood 1	4 weeks	Feed	50.00%
Brood 2	1 week	Feed	25.00%
Brood 3	2 weeks	Litter	50.00%
Pen 1	9 weeks	Chicken	10.00%
Pen 1	9 weeks	Water	100.00%
Pen 1	9 weeks	Litter	100.00%
Pen 1	9 weeks	Feed	25.00%
Pen 2	8 weeks	Chicken	20.00%
Pen 2	8 weeks	Water	100.00%
Pen 2	8 weeks	Feed	50.00%
Pen 3	7 weeks	Chicken	10.00%
Pen 3	7 weeks	Water	50.00%
Pen 3	7 weeks	Litter	100.00%

August 11, 2015

Note: enumerations 1 and 2 for each pen are replicates.

Pen number	Brood/pen age	Isolate type	Percent positive
1	9 weeks	Water enumeration 1	100.00%
1	9 weeks	Water enumeration 2	100.00%
2	8 weeks	Water enumeration 1	100.00%
2	8 weeks	Water enumeration 2	66.67%
2	8 weeks	Chicken	24.00%
2	8 weeks	Litter	80.00%
2	8 weeks	Feed	50.00%
3	7 weeks	Water enumeration 1	66.67%
3	7 weeks	Water enumeration 2	100.00%
3	7 weeks	Litter	80.00%

Polymerase chain reaction lack of results

The purpose of the study was to explore the farm environment for a potential reservoir. To help identify a potential reservoir, the polymerase chain reaction was used. Common serotypes among numerous positive isolates would indicate specific reservoirs for contamination. Unfortunately PCR results always produced inconclusive and nonsensical results. Instead of smooth amplification curves, peaks were jagged and inconsistent. This indicates that some contaminant was present that caused amplification at non-specific sequences. These results were inaccurate and unusable for any comparisons throughout the study. In other instances amplification curves were very small and not steep, indicating that there wasn't enough genetic material for amplification in these isolates. Though this analysis was unsuccessful in identifying a reservoir, the prevalence results provided ample evidence for water fountains and litter being the major environmental reservoirs for *Salmonella* spp. on farm.

Salmonella serotyping

Instead of using PCR results, isolates were sent to the Oregon State Veterinary Science labs to conduct *Salmonella* serotyping. The four isolates were prepared on TSA agar slants. Two isolates were from litter samples of young birds (age 1 day – 1 week) in the preliminary study on March 3. One isolate from March 25 came from the 1 week brood litter (brood 4), and one isolate from May 21 came from the 4 week brood feed (brood 1). In total three different serotypes were determined:

- May 21 feed sample, brood 1 (4 weeks) had the Typhimurium serotype.
- The March 3 pen, litter 1 (1 day) sample had the Poeseldorf serotype.
- The second March 3 pen, litter 2 (1 week) sample had the Braenderup serotype.
- The March 25 brood 4, litter 1 (1 week) sample also had the Poeseldorf serotype, matching the preliminary March 3 serotype results. Specifically, the O and H antigens matched between the two isolates.

Discussion

Individual brood prevalence

Brood prevalence of positive isolates among environmental samples was inconsistent throughout the study. Some results indicated that younger chickens had higher *Salmonella* prevalence than older ones. The sample size on this occurrence was much smaller for all reservoir analyses compared to the other dates, which may have skewed results to indicate higher prevalence at a younger age. To ensure consistent results, the same number of samples should be taken for each brood or pen in future studies.

Salmonella serotyping

Three different serotypes were found from isolates taken throughout the study. Matching isolates were gathered from litter samples on different dates (see above), identified as *Salmonella* Poeseldorf. This serotype has not been associated with any recent outbreaks reported by the Centers for Disease Control (26). Besides this, both serotypes from the isolated strains (page 23) have associations with some foodborne outbreak. *Salmonella* Typhimurium is a very common outbreak serotype that was linked to a multistate outbreak in ground beef in 2013 (27). *Salmonella* Braenderup was linked to outbreak in Natural Foods brand nut butter in 2014 (28). The fact that isolates contained *Salmonella* serotypes known to cause human disease and outbreaks signifies that further treatments must be done to ensure that this farm is not associated with an outbreak.

Control strategies - Water

All results indicated that water was one of the reservoirs of contamination at this facility. Implementation of treatment practices specifically targeting water isolates could be a promising method for reducing *Salmonella* presence in the environment. The World Health Organization developed the Guidelines for Drinking Water Quality precisely to combat the transmission of *Salmonella* in water sources (29). Treatments to prevent transmission could include boiling water and home chlorination, both of which greatly reduced the spread of typhoid fever in India and Uzbekistan (29). More specific treatments include organic acid treatments for the water. Organic acids like formic and propionic acid provides *Salmonella* treatment during feed withdrawal in the pre-slaughter time. This is where susceptibility is much higher to *Salmonella* contamination (30).

While organic acids can inactivate bacterial cells within the water it can lead to corrosion in galvanized pipes. Plant-derived essential oils (like terpenes and terpenoids) can also be used with acids to disrupt the bacterial membrane and improve the microbiocidal effects before feed consumption (30). At 0.15% concentration, a formic and propionic acid product reduced *Salmonella* in water to undetectable levels in 4 hours (30). However, this concentration did not influence *Salmonella* associated with chicks that were artificially challenged by feed with 50 CFU/g of *Salmonella.* Previous studies also showed that existing carriers of *Salmonella* were not susceptible to aqueous acid treatment even when *Salmonella* is eliminated from the water source (30).

Control strategies - Litter

In addition to water, the litter was a likely reservoir of *Salmonella* contamination at the farm. A successful treatment strategy used in previous studies was the pasteurization of the litter with steam and quicklime (31). The quicklime and steam treatment has been used in nurseries to reduce plant pathogens, with the quicklime increasing pH levels beyond what is sustainable for the pathogen (31). Quicklime reacts with water to produce an exothermic reaction that also increases temperature. Treating *Salmonella* Typhimurium-inoculated litter with quicklime at 2.5, 5.0, and 10.0% caused reduction to near undetectable levels in tandem with steam during the study. Steam itself caused a reduction of *Salmonella* Typhimurium by 3 orders of magnitude compared to untreated controls (31). The treatment experiment did not occur in a commercial poultry production factory, but was conducted assuming that future research would confirm its efficacy in that environment.

Reference:

1. United States Department of Agriculture: Economic research service. U.S. per capita availability of beef, pork, and chicken, 1909-2013. Accessed May 2 2016. Web. http://www.ers.usda.gov/data-products/chart-gallery/detail.aspx?chartId=40060
2. North American Meat Institute. The United States meat industry at a glance. Accessed May 2 2016. Web. https://www.meatinstitute.org/index.php?ht=d/sp/i/47465/pid/47465
3. The National Chicken Council. Per capita consumption of poultry and livestock, 1965 to estimated 2016, in pounds. Accessed May 2 2016. Web. http://www.nationalchickencouncil.org/about-the-industry/
4. United States Food and Drug Administration. Poultry Products Inspection Act. Web. http://www.fda.gov/RegulatoryInformation/Legislation/ucm148721.htm
5. United States Department of Agriculture. Guidance for determining whether a poultry slaughter or processing operation is exempt from inspection requirements of the Poultry Products Inspection Act. Accessed April 18 2016. Web. http://www.caes.uga.edu/topics/sustainag/documents/USDAPoultry_Slaughter_Exemption_0406.pdf
6. Gwin, L. New poultry processing options in Oregon. Department of Agriculture & resource economics: Oregon State University. Accessed April 18 2016. Web. http://smallfarms.oregonstate.edu/sfn/su11poultryprocess
7. Oregon Department of Agriculture. Search Active Agricultural licenses. Web. Accessed May 12, 2016. http://oda.state.or.us/dbs/licenses/search.lasso?&division=fsd
8. Oregon Department of Agriculture. Meat products and establishments. Web. Accessed May 2, 2016. http://arcweb.sos.state.or.us/pages/rules/oars_600/oar_603/603_028.html
9. The Centers for Disease Control and Prevention. Multistate Outbreak of Multidrug-Resistant Salmonella Heidelberg Infections Linked to Foster Farms Brand Chicken (Final Update). Accessed April 20 2016. Web. http://www.cdc.gov/salmonella/heidelberg-10-13/
10. Zuraw L. FSIS releases new Salmonella, Campylobacter performance standards for poultry. Food safety news. Accessed May 2, 2016. Web. http://www.foodsafetynews.com/2015/01/fsis-releases-new-salmonella-campylobacter-performance-standards-for-poultry/#.VyhTzRUrKRt
11. Scheinberg, J. A. and C. N. Cutter. 2013. Farmers Market Poultry: A New Perspective on Food Safety. Lancaster Farming. http://lfg.live.mediaspanonline.com/assets/9973221/B07LFWE-110213_1.pdf
12. United States department of agriculture: Food Safety and Inspection Service. Salmonella questions and answers. Accessed April 20, 2016. Web.
13. The Centers for Disease Control and Prevention. Salmonella. Accessed April 20, 2016. Web. http://www.cdc.gov/salmonella/

14. Giannella R. Salmonella. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; 1996. Chapter 21. Accessed May 2, 2016. Web. http://www.ncbi.nlm.nih.gov/books/NBK8435/
15. Bio-Rad. Bacterial serotyping guide for Salmonella. Accessed May 2, 2016. Web. http://www.bio-rad.com/webroot/web/pdf/fsd/literature/FSD_14-0699.pdf
16. United States Department of Agriculture. The USDA Microbiology Laboratory Guidebook. Method 4.08 isolation and identification of Salmonella from meat, poultry, pasteurized egg, and catfish products and carcass and environmental sponges Accessed March 3 2015. Web.
17. Acumedia. Rapport-Vassiliadis broth. Accessed April 20 2016. Web. http://www.neogen.com/Acumedia/pdf/ProdInfo/7730_PI.pdf
18. Acumedia. Tetrathionate broth. Accessed April 20 2016. Web. http://www.neogen.com/Acumedia/pdf/ProdInfo/7241_PI.pdf
19. Begley M., Gahan C., Hill C. The interaction between bacteria and bile. FEMS Microbiology reviews. 2005. 29(4): 625-651
20. Becton-Dickenson. Hektoen enteric agar. Accessed April 20, 2016. Web. https://www.bd.com/resource.aspx?IDX=8970
21. Hudzicki J. Hektoen enteric agar protocol. American Society for Microbiology: Microbe library. Accessed May 3, 2016. Web. http://www.microbelibrary.org/library/laboratory-test/3222-hektoen-enteric-agar-protocol
22. Dmello A, Yotis W. The action of Sodium deoxychoate on Escherichia-Coli. Applied and environmental microbiology. 1987. 53(8): 1944-1946.
23. Becton-Dickenson. Xylose-Lysine-Desoxycholate agar. Web. http://www.bd.com/resource.aspx%3FIDX%3D8991
24. Lin D., Yan M., Lin S., and Chen S. Increasing prevalence of hydrogen sulfide negative Salmonella in retail meats. Food Microbiology. Oct 2014. 43(4): 1-4
25. Chromagar. Chromogenic technology: How does Chromogenic culture media work? Accessed May 2 2016. Web. http://www.chromagar.com/p-Chromogenic_agar_Technology.html#.VxPvm5MrKRt
26. The Centers for Disease Control and Prevention. An Atlas of *Salmonella* in the United States, 1968 -2011. Accessed May 16, 2016. Web. http://www.cdc.gov/salmonella/pdf/salmonella-atlas-508c.pdf
27. The Centers for Disease Control and Prevention. Multistate Outbreak of *Salmonella* Typhimurium Infections Linked to Ground Beef (Final update). Accessed May 16, 2016. Web. http://www.cdc.gov/salmonella/typhimurium-01-13/
28. The Centers for Disease Control and Prevention. Multistate Outbreak of *Salmonella* Braenderup Infections Linked to Nut Butter Manufactured by nSpired Natural Foods, Inc. (Final Update). Accessed May 16, 2016. Web. http://www.cdc.gov/salmonella/braenderup-08-14/index.html
29. Levantesi C., Bonadonna L., Briancesco R., et al. Salmonella in surface and drinking water: occurrence and water-mediated transmission. Food Research International. 2012. 45(2): 587-602.

30. Wales A., Allen V., and Davies R. Chemical treatment of animal feed and water for the control of Salmonella. Foodborne pathogens and disease. 2010. 7(1): 3 – 15.
31. Stingfellow K., Caldwell D., Lee J., et al. Pasteurization of chicken litter with steam and quicklime to reduce *Salmonella* Typhimurium. Journal of Applied Poultry Research. 2010. 19(4): 380 – 386.

Appendix 1

This series of photos (Photo 1 - 6) shows what the environment, chickens, water fountains, and feeders looked like throughout the study.

Photo 1. Chick (about 1 day in age)

Photo 2. Chicks with a feeder for their specific age

Photo 3. Example of water fountain

Photo 4 (1-2 weeks in age)

Photo 5. Chicken age 3-4 weeks

Photo 6. Chickens age 7 weeks +