DISSERTATION

EVALUATION OF RISK FACTORS AND DETECTION OF SELECTED FOODBORNE PATHOGENS ASSOCIATED WITH FRESH PRODUCE

Submitted by

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ABSTRACT

EVALUATION OF RISK FACTORS AND DETECTION OF SELECTED FOODBORNE PATHOGENS ASSOCIATED WITH FRESH PRODUCE

The Economic Research Service (ERS) of the United States Department of Agriculture (USDA) has reported increases of greater than 40 pounds per capita in yearly fresh produce consumption over the last 30 years. Outbreaks associated with fresh produce have also increased with an estimated 46% of foodborne outbreaks attributed to the consumption of various types of fresh produce from 1998 to 2008. One of the foodborne pathogens of concern is *Salmonella* spp., the leading cause of foodborne illness hospitalizations and deaths in the United States (US). *Salmonella* species are ubiquitous microorganisms necessitating increased need for proper surveillance. Testing for major pathogens such as *Salmonella* spp. and *Escherichia coli* O157:H7 in produce is impractical due to large retail volume, variability of contamination, and low sensitivity of current platforms. Irrigation, wash waters, and other agricultural sources offer greater probability for pathogen detection when combined with appropriate sample preparation. One food commodity commonly linked to *Salmonella* spp. outbreaks is tomato. Greenhouse/hydroponic production currently accounts for a large share of tomato production and has had a significant impact on the U.S. fresh-tomato market. There is little known about the possibility of contamination and internalization of foodborne pathogens via greenhouse/hydroponic commercial production since these operations are usually considered relatively sanitary due to the closed environment.

I evaluated the risk factors associated with fresh produce contamination such as contaminated irrigation water and agricultural sources using simple sample preparation, subtyping techniques, and rapid molecular testing. This research is comprised of three study topics: development of an irrigation water concentration method with subsequent detection of *Salmonella* spp. and *E. coli* O157:H7 using Vitek Immuno Diagnostic Assay (VIDAS) technology, comparison of molecular serotyping methods to conventional serotyping methods for *Salmonella enterica* subsp. *enterica* isolates from food and agricultural sources, and evaluation of contaminated irrigation water as a risk factor for contamination of hydroponically grown tomatoes. Novel molecular methods were used in the three studies, including VIDAS UP ® technology, Automated RiboPrinter, Luminex® xMAP *Salmonella* serotyping assay, and pulsed-field gel electrophoresis to detect foodborne pathogens.

Results showed that a novel concentration method was effective in concentration of *Salmonella* spp. and *E. coli* O157:H7 with subsequent detection via mini VIDAS® technology. Molecular serotype methods were unable to serotype isolates obtained from agricultural sources. However, molecular methods allowed us to identify serovars associated with food and clinical sources. *Salmonella* Typhimurium did not survive well in the nutrient solution of a conventional hydroponic system used in tomato production. We also discovered that continuous contamination with *S*. Typhimurium might lead to contamination of the root systems but not contamination of the leaves and fruit. This work illustrates the continuing need to evaluate production methods and pathogen detection techniques to improve the safety of fresh produce.

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CHAPTER 1
Review of Literature

1.1 Foodborne Pathogens and Illnesses

The Centers for Disease Control and Prevention (CDC) has estimated that each year one out six people becomes ill from a foodborne pathogen in the United States (U.S.) resulting in 48 million illnesses, 128,000 hospitalizations and 3,000 deaths (*128*, *151*). There are 31 known foodborne pathogens and although the noroviruses are estimated to cause the most foodborne illnesses, non-typhodial *Salmonella* is leading in hospitalizations and deaths (*128*, *151*). All consumers are at risk but the most vulnerable populations includes the very young, elderly, and those with compromised immune systems (*13*). Based on previous foodborne outbreaks, many factors contribute to bacterial contamination and proliferation such as cross contamination, inadequate temperature control, unsafe handling or insufficient cooking (*24*). Agricultural animals are reservoirs and have a major role in the spread of these foodborne pathogens which are dispersed through food and water supplies after contact with contaminated feces. Many of these pathogens are considered enteric microorganisms that are able to colonize via the mouth and gastrointestinal tract of humans, food animals, and birds (*17*, *49*, *53*). Foodborne pathogens are a major issue in the food industry due to their ability to survive during transportation or storage for long-time periods (*89*, *106*). Eight pathogens consistently associated with significant health problems worldwide are *Campylobacter jejuni*, *Salmonella enterica*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli* O157:H7, Shiga-toxin producing *E. coli* strains (non-O157 STEC) and *Vibrio* spp. (*164*).

1.2 Escherichia coli O157:H7

Escherichia coli O157:H7 is a Gram-negative, facultative anaerobic bacterium that benignly inhabitants the gastrointestinal tracts of many warm-blooded animals species including cattle (*108*). The serotype O157:H7 along with several other serotypes are referred to as enterohaemorrahagic *E. coli* (or EHEC) and accounts for over 90% of all cases of hemorrhagic colitis (HUS) in industrialized countries (*96*). This bacterium produces *shiga*-like toxin which causes severe/chronic distinct syndromes of diarrhea diseases such as hemorrhagic colitis (HUS), abdominal cramps, and bloody stool but little to no fever (*6*, *16*, *88*, *117*). EHEC O157:H7 is one of the 25 top foodborne pathogens actively under surveillance primarily due to the more than 60,000 cases reported every year in the U.S. (*128*). According to the U. S. Food and Drug Administration (FDA), the infectious dose of *E.coli* O157:H7 is estimated to be 10-100 cells (*144*).

Cattle are a major reservoir for *E. coli* O157:H7, although it does not cause illness in cattle and beef products are a common source of human infection (*6*, *16*, *26*, *49*). Environmental studies have indicated that the bacterium can persist in manure, soil, water troughs, contaminated seeds, and other farm locations which is an important implication for the persistence of the pathogen in cattle herds and contamination of agricultural water and crops (*6*, *26*, *96*). Other species of livestock, domestic, and wild animals such as deer, sheep, goats, horses, dogs, and birds provide a specific niche for the bacterium as well (*26*, *96*). Pathogenic strains of *E. coli* can survive in the open environment where the ability to use available nutrients and attaching to surfaces plays a critical role in survival (*26*).

E. coli O157:H7 is transmitted to humans in various ways specifically through food, water, or direct person-to-person contact. Pathogens from bovine origin have been implicated in

numerous outbreak investigations with a variety of food sources identified such as apple cider, raw milk, radish sprouts, lettuce, alfalfa sprouts, and drinking water (*15*, *26*, *67*, *96*). It was first recognized as a human pathogen in the 1980s following two large outbreaks of gastrointestinal illness in the United States associated with undercooked ground beef (*49*, *83*, *122*, *154*). In 1994, the United States Department of Agriculture (USDA) declared *E. coli* O157:H7 an adulterant of ground beef which resulted in the implementation of mandatory recalls (*13*). Since the early 2000s, *E. coli* O157:H7 has also been linked to contamination associated with fruit and vegetables and has been implicated in a growing number of recognized outbreaks (*96*).

1.3 Salmonella

Salmonella is a Gram-negative, facultative anaerobic, rod-shaped, non spore-forming, motile bacterium which harbors asymptomatically in the gastrointestinal tract of agricultural animals (*49*). Due to *Salmonella*'s ubiquitous nature, it is resilient and can adapt to extreme environmental conditions (*49*). *Salmonella* is the leading cause of severe foodborne cases resulting in 35% of hospitalizations and 28% of deaths in the United States (*128*). There are two species of *Salmonella*: *bongori* and *enterica*. *S. bongori* has no subspecies. However, *enterica* is commonly associated with foodborne outbreaks and over 2600 serovars have been identified (*57*). *S. enterica* is subdivided into six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) (*57*). The disease caused by *Salmonella*, salmonellosis, can manifest into two distinct diseases: typhoid-like-fever and self-limiting gastroenteritis which leads to diarrhea, stomach cramps, and occasional vomiting and fever (*49*). The infectious dose of *Salmonella* is as few as 15-20 cells (*144*).

Salmonella has an agricultural animal origin which facilitates dispersal of the pathogen through feces into the environment and often leads to contamination of other wild and farmed

animals and feed crops (*49*). Various groups of animals species that have been infected by *Salmonella* include sheep, goats, cattle, chickens, pigs, birds, and even reptiles and amphibians (*3*, *16*). Many of the serovars are host specific while some are restricted to one kind of animal such as serovar Pullorum and chickens (*34*). However, serovars Typhimurium and Enteritidis are broad range and considered hazardous because they are responsible for high numbers of humans illnesses and infections in animals (*27*, *34*).

Salmonella may be introduced to food under various circumstances and has an long standing association with poultry and poultry products (*3*). Since the 1960s there has been an increase in isolation of non-host specific *Salmonella* in poultry products and human cases (*24*). *Salmonella enteric* serovar Enteritidis is primarily linked to the consumption of poultry, eggs, and egg-derived products (*24*). Poultry products today remain a major source of *Salmonella* however, other meats such as pork and beef have been identified as sources of salmonellosis (*3*, *24*). For example, there are pre-harvest concerns for pork due to *Salmonella*'s ability to colonize on the farm in the feed, water, and other environmental habitants (*3*, *24*). Recently *Salmonella* has been linked to fresh produce outbreaks with vegetables such as cilantro, broccoli, cauliflower, lettuce, tomatoes and spinach (*24*).

1.4 Foodborne Pathogens and Fresh Produce

Recent foodborne outbreaks associated with consumption of fresh produce has raised concerns regarding these products as sources of foodborne infections (*31*, *49*). There has also been an increase in consumption of fresh produce due to the greater demand for having access to fresh produce year-round (*23*, *31*, *74*). Fresh and fresh-cut produce requires minimum processing which means no elimination steps are used for either natural or hazardous microorganisms on fresh produce (*62*). Risk factors evaluated during the spread of foodborne

pathogens to fresh produce include: how it is grown, harvested, packed, processed, transported, distributed, prepared, and consumed (*4*). Possible sources of contamination which spread foodborne pathogens to fresh produce include: irrigation water, manure, wastewater, and direct contact with food handlers (*89*, *106*, *143*). Fecal contamination from wild and domestic animals via agricultural irrigation water or runoff also plays a role in the spread of contaminants to fresh produce (*4*, *14*, *26*, *106*). Many of the foodborne outbreaks worldwide attributed to fresh produce from 2005 to 2011 involved *Salmonella* spp., *Escherichia coli* O157:H7, and *Listeria monocytogenes* (*110*).

Previous studies have evaluated the ability of foodborne pathogens to be internalized in food crops, however the mechanism of action is not fully understood (*31*, *38*, *52*, *56*, *59*, *65*, *80*, *97*, *132*, *135*, *165*). Internalization of *E. coli* O157:H7 was detectable after cultural enrichment of spinach and lettuce seedlings when germinated within a growth chamber in autoclaved and non-autoclaved soil at contamination levels of 2.0 and 3.8 log CFU/g respectively (*38*). A lack of internalized *E. coli* O157:H7 was also observed in both lettuce and spinach germinated from seedlings in contaminated soil in the field where it was suggested that the pathogens were in competition with indigenous soil bacterial and environmental stress was greater in the field than the growth chamber. Using high-resolution microscopy researchers have examined colony formation of *E. coli* O157:H7 in roots and within the internal tissues of both lettuce and spinach (*160*). The researchers also discovered colonization of the pathogen in the apoplast of the root located between plant cells. Colonies were also detected inside the cell wall of epidermal and cortical cells of the spinach plants, and sporadic cells of the laboratory strain of *E. coli* K-12 were found on the spinach with no evidence of internalization. *E. coli* O157:H7 was exposed for 9 weeks and survived on lettuce leaves and in soil longer during the fall season in comparison to

the spring season (*111*). It was also observed that *E. coli* O157:H7 could be transferred from the soil and irrigation water during pre-harvest conditions. Survival of *E. coli* O157:H7 after treatment with the fungicide azoxystrobin (Quadris) at the highest concentration (2.66%) were found to be significant with an increase in cell population after 6 hours incubation and colonies continued to grow after 24 hours incubation (*33*).

Post-harvest packaging and temperature may also play a role in the survival of *E. coli* O157:H7 on fresh-cut fruits and vegetables. Researchers found that modified atmosphere packaging (MAP) did not affect the growth of *E. coli* O157:H7. However, at 5°C *E. coli* did not grow but it did survive throughout the study and at 25°C *E. coli* grew on escarole, carrots, and melon but not on fresh-cut pineapple (*1*).

Microscopic analysis has been used to examine the variation of *S*. Typhimurium internalization and it was found that 2 samples (iceberg lettuce and arugula) had high incidence, 3 (romaine and red lettuce, and basil) had low to moderate incidence, and 2 samples (parsley and tomato) exhibited low incidences (*52*). *Salmonella* has also been found to colonize in the plant and edible portions of green onions (*105*). Tomatoes have been identified as a vehicles for *Salmonella* (*112*). *Salmonella* and *Escherichia coli* contamination was observed after surveillance from 2003 to 2004 of a greenhouse hydroponic farm where two natural events occurred with water runoff and presence of wild animals. Presence of both pathogens was found from tomato samples, water puddles, soil, shoes, and feces of the local wild and farm animals. Specifically, *Salmonella* serotypes Montevideo, Newport and F serogroup linked to the tomato samples were isolated from goat feces and personnel shoes (*112*). Tomato plants have been evaluated for survival of *Salmonella* pre and post fruit development where it has been found that less than 50% of inoculated pre and post flower development samples were positives (*60*). In

this same study, *Salmonella enterica* was also found to be highly prevalent on the surface and stem scar tissue of the tomato plants and the serotype Montevideo was isolated most frequently from samples.

1.5 Detection of Foodborne Pathogens

1.5.1 Isolation of Foodborne Pathogens

Traditional isolation methods for *Salmonella* in food include non-selective pre-enrichment, selective enrichment followed by selective/differential plating, and confirmation by biochemical and serological analysis which are detailed in the Bacteriological Analytical Manual (BAM) published by the U.S. Food and Drug Administration (FDA) (*12*, *92*, *146*, *147*). *Salmonella* isolation for food involves pre-enrichment with non-selective media such as buffered peptone water (BPW), selective enrichment with Rappaport-Vassiliadis (RV) medium and Tetrathionate (TT) broth, and plating on selective agar Hektoen enteric (HE), Xylose lysine desoxychlate (XLD), and Bismuth Sulfite (BS) agars (*147*).

1.5.2 Traditional Serotyping of *Salmonella* spp.

Slide agglutination is used to phenotypically serotype *Salmonella* isolates based on the White-Kauffmann-Le Minor scheme. This method has been in existence for more than 80 years and is often used during an outbreak investigation to characterize isolates to the subspecies level, with over 2600 serotypes of *Salmonella* identified (*70*, *157*). Anti-sera is used to determine the antigenic variability of *Salmonella* surface structures, including lipopolysaccharides (O antigen), flageller protein (H antigen), and capsular polysaccharides (Vi antigen) (*64*, *118*, *142*).

Traditional serotyping has been estimated to correctly serotype about 90% of common *Salmonella* clinical isolates (*156*). However, there are some disadvantages to this method such as being labor intensive and time consuming depending on the number of isolates. The need for greater than 300 different anti-sera for preparation and the quality control techniques require well trained technicians (*64*, *121*, *133*, *164*). There are limits to the discriminatory power and reproducibility due to false-negative reactions with weak-positive nonspecific agglutination and the reaction is based on expression of particular genes on the surface (*100*, *133*). Nevertheless, during an outbreak investigation slide agglutination is followed by further discriminatory approaches including molecular subtyping (*158*).

1.5.3 Molecular Methods

Rapid and subtyping methods for foodborne pathogens detection are being developed which will improve the capability to differentiate subtypes of foodborne pathogens. These methods should have the ability to be very specific, exhibit high sensitivity and discriminatory power and therefore provide better standardization and allowing faster and better reproducibility during outbreak investigation (*64*, *133*, *164*). Many of these methods are considered molecular and are classified into these categories: nucleic-acid-based methods, immunological methods, and biosensor methods (*7*, *121*, *164*, *166*).

1.5.4 Automated Immunoassay

The Vitek Immuno Diagnostic Assay System UP Phage Technology assay (VIDAS UP PT, bioMérieux, Inc., Durham, NC USA) is an example of an enzyme-linked fluorescent assay (ELFA) that uses recombinant phage proteins (*37*, *54*, *67*, *88*, *107*, *131*). Kits have been

developed to detect *E. coli* O157:H7, *Salmonella* spp., and *Listeria* spp. This system has a two-step assay that combines a cocktail of monoclonal antibodies and recombinant bacteriophage proteins, and targets somatic and flagellar antigens, allowing detection of motile and non-motile strains. The methodology involves an aliquot being placed in a reagent strip which includes washing solutions with specific anti-pathogen proteins conjugated to alkaline phosphatase and substrate (*107*, *131*). Next, the reagent strip is heated and then placed in the mini-VIDAS platform. Second, the sample aliquot is transferred through the platform by a disposable solid phase receptacle (SPR) coated with antibodies to each stage of the reagent strip until detection by fluorescence generated by a reaction of alkaline phosphatase and substrate. Advantages of the VIDAS UP PT technology include ease of use and enhanced sensitivity when samples have been incubated to increase bacterial number after 18-26 hours of enrichment (*19*, *67*, *88*).

The capability of VIDAS UP SPT (*Salmonella* Phage Technology) and fluorescence *in situ* hybridization (FISH) were compared to the International Organization for Standardization Method 6579 (ISO) for detection of *Salmonella* spp. from artificially inoculated beef, pork, and poultry meat samples (*163*). Both VIDAS UP and FISH detection results agreed with ISO with relative specificity, accordance, and sensitivity rates of 90%, 96.3%, and 100%, respectively for VIDAS UP detection. Detection of *Salmonella* spp. using VIDAS SPT was compared to the USDA/FSIS microbiology laboratory guidebook (MLG) reference method in a multi-laboratories study on artificially inoculated raw ground beef at two testing portion sizes (25 and 375g) (*19*). The raw ground beef test portions were inoculated at low and high levels of inoculation and uninoculated samples served as controls. The researcher from this study recommended that VIDAS SPT method along with optional ASAP and IBISA agar confirmation method could be

adopted for Official First Action status for the detection of *Salmonella* in a variety of foods and environmental samples.

The capability of detection using VIDAS UP ECPT (*E. coli* Phage Technology, including H7), BAX PCR system PCR assay and VIDAS ECO were compared in artificially contaminated raw beef meat, raw milk, raw chicken, soybean, sprouts and fresh papaya juice (44). Various concentration suspensions were analyzed using three detection methods and it was found that VIDAS ECPT UP was able to detect all of the 18 *E. coli* O157:H7 strains at the suspension level of 4 log CFU/mL. The VIDAS ECPT UP was also able to detect all 18 of the raw beef, raw milk, and raw chicken samples after 6 hours of enrichment. After 8 hours of enrichment, all 18 samples were detected for both soybean sprouts and fresh papaya juice.

Detection of *E. coli* O157:H7 in artificially inoculated raw ground beef was compared using the VIDAS UP ECPT, Real-Time PCR and the USDA-FSIS reference methods (63). The shortest enrichment time that allowed for optimum detection in single samples (25g) was evaluated and it was determined that 6 hours of enrichment in buffered peptone water (BPW) at 41.5 °C was sufficient for detection in ground beef. The results were comparable to the USDA-FSIS reference method with enrichment by modified tryptic soy broth (mTSB). For composite samples (375g), the sample type, sample-to-broth ratio, and strain did not affect the detection via both VIDAS ECPT UP and the RT-PCR method where positive results were observed after 24 hours of enrichment.

1.5.5 Molecular subtyping methods

1.5.6 Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) is a molecular subtyping method commonly used at state public laboratories in conjunction with CDC's surveillance program (*45*). The CDC national database Pulse Net is a reference database which uses PFGE patterns and compares within networks using standardized protocols (*45*, *166*). The methodology involves pure culture cells embedded into standard electrophoresis agarose or acrylamide gels, lysing cells to release genomic DNA, rare cutting restriction enzymes to produce large fragments which reflect the DNA sequence of the whole genome, and finally separation of DNA fragments are separated using alternating electric fields with sizes representing up to 2000 kb (*44*, *133*).

PFGE analysis was performed using restriction enzyme *Xba*I and *Bln*I on both human and food animal non-typhodial *Salmonella* isolates (62). The 20 most common human *Salmonella* patterns representing 39% of the human isolates from Pennsylvania were serotyped from those isolates. Among the serotypes represented were Berta, Enteritidis, Heidelberg, Newport, Thompson, Typhimurium (including variant 5-) and one antigenic formula (1 4, [5], 12:i:-). Serotypes Enteritidis and Kentucky patterns were the most common PFGE pattern from the human and food animal *Salmonella* isolates, respectively. The shared common patterns for the food animal *Salmonella* isolates were recovered from chickens and the common source PFGE pattern was Heidelberg pattern followed by Typhimurium which was primarily isolated from swine.

PFGE analysis on a representative group of *Salmonella* isolates from fecal, feed and environmental samples were collected from 32 different wild and exotic animal species in

captivity and their environment in Ohio (23). DNA digestion was performed using restriction enzyme *Xba*I. The serotypes frequently observed were Typhimurium (63.4%), Newport (32.1%), and Heidelberg (5.3%) which are also serotypes of public health significance. There was also a high occurrence of *Salmonella* in the fecal samples with a very diverse serotype population of at least 5 common serovars, primarily Typhimurium and Newport. Only one serovar was observed in the environmental isolates (Heidelberg) and two serovars for the feed isolates (Heidelberg and Agbeni).

1.5.7 Automated RiboPrinting

Automated RiboPrinting is a subtyping method based on digestion of rRNA gene restriction fragments polymorphisms which creates a pattern that is compared to a database (*7*, *103*). The methodology involves picking a pure colony, suspended in sample buffer, and heat-treating before it is placing in the automated RiboPrinter Microbial Characterization System (DuPont, Wilmington, DE). Once in the system, the sample is treated with a lysing agent, which releases DNA. The DNA is digested with a restriction enzyme (*Pvu*II for common *Salmonella* serotypes and *Eco*R1for *E. coli* O157:H7)(*7*). The restriction fragments are then transferred and separated by size in an agarose gel cassette. Using the Southern blot analyses, the DNA fragments are transferred to a nylon membrane. Following denaturation, the membrane is hybridized with chemically labeled DNA probes complementary to ribosomal sequences, yielding a Riboprint pattern. The membrane is washed and the Riboprint patterns are captured using a charged-coupled device (CCD) camera. The Riboprint patterns are matched to reference patterns using DuPont® RiboExplorer Software, a customized software system. The advantage of the automated RiboPrinter system is that it offers speed, simplicity of operation and high

reproducibility due to the method analysis occurring in a single unit (*7*, *133*). The automation of the ribotyping method alleviated many shortcomings of traditional ribotyping methods and the entire method from cell lysis to image analysis was performed in 8 hours (*166*).

Automated RiboPrinting with restriction enzyme *Pvu*II was used to confirm results generated from Dot blot for genetic characterization of *S.* Enteritidis strains from poultry products and environmental samples (*73*). Selected bacterial cultures with the serotypes of Enteritidis, Berta, Maarseen, Typhimurium, Pullorum, Arizonae/III, and Heidelberg were used in the study. The RiboPrinter was able to confirm the serotypes of Enteritidis, Typhimurium, Arizoniae and was not able to identify serotypes Berta (as Bareily), Maarseen (as California), Pullorum (as PvuII Group II), and Heidelberg (as Typhimurium). A variety of 259 *Salmonella* poultry isolates consisting of 32 unique serotypes or subtypes were generally characterized using the automated RiboPrinter (*7*) . All of the isolates were confirmed using the USDA serotyping method. There was an overall agreement of riboprinting identification with 208 of the 259 (80%) isolates being confirmed with the USDA serotyping method. A group of 27 *S.* Montevideo isolates were not confirmed using the USDA method. When that group was removed the ribotype agreement with serotypes was 207 out of the 231 isolates (90%). Serotypes that also exhibited poor correlation between serotyping and ribotypng identification were Agona, Inverness, Ouakum, and Tennessee. There were also serotypes that gave multiple ribotype identifications such as 4,5,12, I-monophasic Typhimurium, Heidelberg, Schwarengrund, and Typhimurium. Automated RiboPrinting has also been used for genetic characterization of *Salmonella.* spp. strains collected from dairy cows, calves, and the farm environment (*115*). The RiboPrinter System characterized the 61 isolates into 12 serotypes including Senftenberg, Enteritidis, Typhimurium, Gallinarium, Java, Hartford, Infantis, Pullorum, Arizonae/III, Havana,

SaintPaul, Lexington, and other *Salmonella*. Automated RiboPrinting has also been used to confirm the genetic characterization of strains of the top 20 *Salmonella* serovars among U.S. human sources, the top 20 serovars among U.S. nonhuman sources, and the top 20 serovars among nonclinical nonhuman sources (*121*). The RiboPrinter System predicted 34 of the 46 (74%) serovars that were congruent with traditional *Salmonella* serotyping results. The serotypes incorrectly identified were 4,5,12:i:-, Braenderup, Give, Javiana, Muenster, Orion var 15+34+, Uganda. Serovars not identified were Blockley, Dubin, Montevideo, Typhi, and Typhimurium var. 5-.

1.5.8 Luminex xMAP *Salmonella* Serotyping Assay

The Luminex® xMAP assay (Luminex, Austin, TX) is another molecular method for detection of infectious microorganisms which is widely applied in healthcare, water quality, food industries and currently used in federal agencies (*35*, *43*, *82*, *85*, *95*, *153*). This technology is a multiplexing system incorporating polystyrene microsphere beads that are internally dyed with precise amounts of multiple spectrally distinct fluorochromes, which allows an array of detection of 500 different analytes in a single tube/reaction. This methodology involves the addition of a single colony mixed with a buffer solution. Samples are heated, allowed to cool, suspended in a buffer and debris removed by centrifugation to release genomic DNA. The genomic DNA is amplified using the gene sequence. The amplicon is hybridized to the target fluorochrome specific microspheres beads. An additional fluorochrome coupled with a reporter molecule is added prior to analyses using the reporter system (Bio-Plex 200 System operating using Bio-Plex Manager software, Hercules, CA). Detection of specific amplicons is interpreted according to the median fluorescence intensity (MFI) score assigned for each sample by the Bio-Plex system.

Fitzgerald et al. (2007) and McQuinston et al. (2011) developed the Luminex xMAP® *Salmonella* serotyping assay (SSA) which consists of three separate tests with sufficient sensitivity to determine O and H antigens simultaneously as well as serotype specific markers in the additional targets (AT) test (*36*, *43*, *95*). Fitzgerald et al. (2007) used a panel of isolates from the *Salmonella* Reference Collection in the Foodborne and Diarrheal Diseases Branch of the CDC. The *Salmonella* serogroup O13 *rfb* gene region was selected for detection of the six most common serogroups in the United States (B, C1, C2, D, E, and O13) plus serotype Paratyphi A which is directly involved in O-antigen biosynthesis. Luminex ® microsphere beads were used for detection and the reading of the Mean Fluorescent Intensity (MFI) was performed using the Bio-Plex platform. The validation of the O-group panel revealed that 362 of the 384 (94%) isolates were correctly identified and compared to the traditional method. Seventeen of the remaining isolates (4.4%) produced results consistent with what is known about the molecular basis for serotypes but different from the results of traditional serotyping and the remaining 5 isolates were generated as false-negative results. McQuinston et al. (2011) used a panel of isolates from the CDC collection at the National *Salmonella* Reference Laboratory to develop a DNA-based assay targeting the genes encoding the flagellar antigens (*fliC* and *fljB*). The target assays were developed for fifteen H antigens, 5 complex major antigens and 16 complex secondary antigens. The Kauffmann-White serotyping scheme was used in the development of the assay. The validation of the H-antigen panel revealed that 461 of the 500 (92.2%) isolates were correctly identified when compared to traditional methods. The isolates correctly serotyped included partially serotyped and monophasic or non-motile strains that possessed flagellar genes. The probe was not included for some flagellar genes, which were probable for detection. False-negative reactions were observed and 39 strains were not correctly identified because they did

not possess antigens that could be detected by this assay. The authors suggested that the method could enhance the ability of clinical and public health laboratories for serotyping *Salmonella*.

The FDA used the Bioplex as well as the Luminex® platforms to develop a xMAP assay to identify the O serogroup and H antigens of *E. coli* O157:H7 (*82*, *85*). The Luminex® platform has also been used to detect *Avian Coronavirus* (also known as Infectious bronchitis virus) in poultry (*123*). The Luminex xTAG analyte-specific reagent platform has been used on diarrhea-causing pathogens, such as *Campylobacter jejuni*, *Salmonella* spp. *Shigella* spp., Shiga toxin-producing, *E. coli* O157:H7, and other STEC *E. coli* (*104*). The Luminex® xTAG beads were used to develop a rapid method to detect *gyr*A, *gyr*B, *par*E genes of *S.* enterica serovars Typhi and Paratyphi A that are results of nalidixic acid resistance (NalR) and/or decreased susceptibility to fluoroquinolones (*136*).

Overall Objectives

The following studies evaluated risk factors associated with fresh produce contamination such as contaminated irrigation water, food and agricultural sources, using simple sample preparation, molecular subtyping techniques, and rapid detection methods.

CHAPTER 2
Development and Evaluation of Large Volume Irrigation Water Sampling and Rapid Detection Protocol for *Salmonella* spp. and *Escherichia coli* O157:H7

2.1 Summary

Testing of fresh produce for major pathogens such as *Salmonella* spp. and *Escherichia coli* O157:H7 is impractical due to small sample sizes, variability of contamination, and low sensitivity of current platforms. Irrigation or process water with inadequate microbial quality has been identified as a potential source of contamination of fresh produce but has greater probability for pathogen detection when combined with appropriate sample preparation and concentration methods. The purpose of this study was to develop an integrated method for the sensitive and specific detection of *E. coli* O157:H7 and *Salmonella* spp. from irrigation water by combining a bacterial concentration and sample preparation step (Modified Moore Swab -MMS and Disposable Inline Filter - DIFs) with specific detection via the VIDAS UP PT technology. Three-strain cocktails of *Salmonella* spp. or *Escherichia coli* O157:H7 strains associated with produce outbreaks were used to contaminate large volume samples of irrigation water (10 L) at concentrations of 0, 1, 10, and 100 CFU/10L which was used to assess the efficacy of MMS and DIF for *Salmonella* spp. and DIF for *E. coli* O157:H7. For MMS concentration of *E. coli* O157:H7, irrigation water was spiked at levels 0, 1, 10, and 1000 CFU/10L. Samples were enriched pre- and post- concentration in selective media for up to 18 hours at 42°C. Samples were collected at 0, 8, 12, and 18 hours of enrichment. *E. coli* O157:H7 was detected at concentrations of 1000 and 1 CFU/10L for MMS and DIF, respectively, following enrichment for 8 hours. *Salmonella* spp. was detected at concentrations of 10 and 1 CFU/10L following

concentration by MMS and DIF, respectively, and enrichment for 18 and 12 hours, respectively. The pre(non)- concentrated samples were not detectable using the mini-VIDAS technology. The results of this study show that mini-VIDAS technology can be used to sensitively and specifically detect *Salmonella* spp. and *E. coli* O157:H7 in large volumes of irrigation water when a pre-concentration step is used. The concentration protocol developed employed shortened time-to-detection and improved sensitivity of previously established methodology.

2.2 Introduction

Foodborne outbreaks attributed to fresh produce continue to be a concern worldwide. For example, between 1998-2008, produce was estimated to cause 46% of foodborne outbreaks in the United States (*113*). Leafy greens (22.3%) and fruits & nuts (11.3%) accounted for 2 of the top 3 food groups associated with foodborne outbreaks during that time (8). Small sample sizes, variability of contamination, and low sensitivity of current methods are some of the setbacks that make detection of foodborne pathogens in fresh produce impractical (*22*, *93*, *126*).

Irrigation or process wash water with inadequate microbial quality has been identified as a potential source of contamination of fresh produce (*46*, *72*, *140*). Surface water used as an irrigation source poses a great risk for contamination with pathogenic microorganisms due to high probability of contact with fecal material from domestic or wild animals (*46*, *140*). Reusing processing water is also a concern because it may result in microbial contamination or cross contamination of crops (*46*). Filtration of large volumes of water can be used as an alternative method to effectively concentrate human enteric pathogens in irrigation water (*22*, *93*, *126*). The need to concentrate large volumes of irrigation water due to the low concentrations of pathogens presents a major obstacle of testing that needs to be addressed to be able to detect presence of foodborne pathogens.

We previously reported on the use of Modified Moore Swabs (MMS) as a method to concentrate bacterial foodborne pathogens from large volumes of irrigation water (*20*, *22*, *28*, *93*). The MMS is comprised of a plastic or polyvinyl chloride (PVC) cassette filled with rolled up cotton gauze which functions as a coarse filter and has the capability to trap bacteria as water is filtered through (*22*, *93*). The MMS has been effectively used to detect food and water-borne contaminants such as *E. coli* O157:H7 and *Salmonella* spp. from large volumes of irrigation and greenhouse water sources (*20*, *22*, *93*, *126*). Commercially available Disposable Inline Filter-30 (DIF-30, United Filtration systems™) have also been evaluated for concentration of foodborne pathogens (*28*). These filters consist of a welded housing with encapsulated microfiber filter elements for high efficiency with gas and liquid filtration (*149*). The 0.3-micron filter pore size facilitates concentration of human enteric pathogens. Advantages of the DIFs are compatibility with portable analyzers and ease of replacement making them well suited for field-based studies.

Several rapid immunological methods for isolation and detection of *E. coli* O157:H7 and *Salmonella* spp. have been commercialized and are based on targeting exposed cell surface proteins. Such methods include immunomagnetic separation, latex agglutination, enzyme linked immunosorbent assays (ELISAs), and lateral flow assays (*109*). False positive results may be observed when using immunological assays to test foods for the presence of *E. coli* O157:H7 and *Salmonella* due to denaturation or degradation of the capture antibody, as well as non-specific binding of the detection antibody to the denatured capture antibody (*109*). Alternatively, false positive test results occur due to cross-reactivity with surface associated epitopes from closely related bacteria such as *Citrobacter* spp. and *Hafnia* spp., which are often present in plant material (*79*, *163*).

Other affinity methods can be used as an alternative to the use of antibodies to detect foodborne pathogens such as the VIDAS UP PT (Phage Technology) assay (bioMérieux, Inc., Durham, NC, USA) which is an enzyme-linked fluorescent assay (ELFA) that uses recombinant phage proteins to specifically detect *E. coli* O157:H7, *Salmonella* spp. and *Listeria* spp. (*37*, *54*, *67*, *88*, *107*, *131*). One advantages of the VIDAS UP PT technology is enhanced sensitivity when samples have been enriched for 18-26 hours to increase bacterial concentration (*19*, *67*, *88*). We hypothesized that a pre-concentration step prior to enrichment would further increase assay specificity. The objective of this study was to develop an integrated method for the sensitive and specific detection of *E. coli* O157:H7 and *Salmonella* spp. from irrigation water by combining a bacterial concentration and sample preparation step (MMS and DIFs) with specific detection via the VIDAS UP PT technology.

2.3 Materials and Methods

2.3.1 Bacterial Strains

The bacterial strains used in this study were associated with fresh produce outbreaks and included *Escherichia* coli O157:H7 PTVS016 (lettuce outbreak), *E. coli* O157:H7 PTVS087 (lettuce isolate), *E. coli* O157:H7 PTVS088 (lettuce isolate), and *Salmonella* enterica subsp. enterica serovar Montevideo MDD22 (tomato-associated outbreak), serovar Poona MDD237 (cantaloupe-associated outbreak) and serovar Newport MDD314 (tomato-associated outbreak environmental isolate). The *E. coli* isolates were obtained from Dr. Trevor Suslow at the University of California, Davis, CA USA. The *Salmonella* isolates were obtained from Dr. Michelle Danyluk at the University of Florida, Gainesville, FL USA. Stock cultures were made and maintained in 20% glycerol and were frozen at -80°C.

2.3.2 Inocula and Sample Preparation

Bacterial inoculums were prepared by growing bacterial cultures from -80°C glycerol stock streaked onto Tryptic Soy Agar (TSA, Difco Detroit, MI). An isolated colony was transferred to a test tube containing 10 mL of Tryptic Soy Broth (TSB, Difco Detroit, MI, USA). Broth cultures were then incubated at 37°C for 16 to 18 hours with shaking. Two separate 3-strain cocktails of *E. coli* O157:H7 and the *Salmonella* serovars were prepared by combining equal concentrations of overnight cultures that were then serially diluted in lambda buffer (100mL NaCl, 8mM MgSO4 • 7H2O, 50mL Tris-HCl [pH7.5]). Irrigation water was obtained from the Big Thompson River (40°25'18.6"N; 105°13'36.7"W [40.421834,-105.226869] Loveland, CO, USA) in 20 liter Nalgene™ carboys (Thermo Fisher Scientific, Waltham, MA, USA) and stored for no longer than 24 hours at 4°C prior to use. To prepare artificially contaminated irrigation water samples, 1 mL of the bacterial inoculum was used to spike 10-liter samples. For *Salmonella* spp. MMS and DIF concentrations spiking levels were 0 (negative control), 1, 10, and 100 CFU/10L. *E. coli* O157:H7 DIF concentration, was spiked into the irrigation water at 0 (negative control), 1, 10, and 100 CFU/10L. *E. coli* O157:H7 MMS concentration, was spiked into the irrigation water at 0 (negative control), 1, 10, and 1000 CFU/10L. Spiking levels were determined by plate count.

2.3.3 Concentration

All spiked irrigation water samples were thoroughly mixed prior to the concentration process. In addition, a 25 mL aliquot of the spiked irrigation water was collected for pre-concentration analysis from all 10 L samples. For each 10 L spiked irrigation water sample, bacterial concentration was conducted using either a MMS or a Disposable inline filter-30 [DIF-

IN30] (United Filtration System, Sterling Heights, MI, USA) that was connected to a peristaltic pump. The MMS cartridge and gauze swab were prepared as described by (*22*). The samples were concentrated at a flow rate of 500 mL/min(*11*). Following concentration, the cotton gauze from the MMS was removed and placed in a stomacher bag (Nasco Whirl-Pak, Fort Atkinson, WI, USA) and collected as the post-concentration sample. The intact DIF was collected as the post-concentration sample.

2.3.4 Enrichment

For enrichment of pre-concentration samples, 25 mL of spiked irrigation water was added to 225 mL of buffered peptone water which contained a selective enrichment supplement. The selective enrichment supplement for *E. coli* O157: H7 was prepared by adding 8 mg/L vancomycin (Sigma Scientific, Saint Louis, MO, USA). The selective enrichment supplement for *Salmonella* spp. was prepared by adding the *Salmonella* supplement® for the VIDAS UP PT assay according to the manufacturer's instruction (bioMérieux, Inc., Durham, NC, USA). For the post-concentration samples, 225 mL of buffered peptone water containing the respective selective enrichment supplement was added to the stomacher bag which contained the MMS cotton gauze. For enrichment of the DIF samples, 25 mL of buffered peptone water containing the respective selective enrichment supplement was introduced into the DIF filter and both ends of the filter were sealed using parafilm. This allowed enrichment of the DIF samples to occur directly within the DIF cartridge. Samples were enriched at 42°C for up to 18 hours, and aliquots were removed at 0, 8, 12, and 18 hours of enrichment for analysis.

2.3.5 Detection

The VIDAS UP PT method consists of a two-step assay that combines a cocktail of monoclonal antibodies, and recombinant bacteriophage proteins, which target somatic and flagellar antigens, allowing detection of motile and nonmotile strains. The mini- Vitek Immunodianostic Assay System (miniVIDAS) (bioMérieux, Inc., Durham, NC, USA) apparatus detects the intensity of fluorescence generated from the enzyme linked fluorescence assay. Both pre and post concentration samples were analyzed on the miniVIDAS apparatus using the VIDAS UP® *Salmonella* (SPT) or VIDAS UP® *E. coli* (including H7) kits (bioMérieux, Inc., Durham, NC, USA), according to the manufacturer's instructions.

2.3.6 Confirmation

All *E. coli* O157:H7 or *Salmonella* spp. presumptive positive mini VIDAS results were confirmed using real time polymerase chain reaction (RT-PCR) assays and plating on selective media. For RT-PCR, nucleic acid isolation was performed using the MicroSEQ *E. coli* O157:H7 and *Salmonella* spp. Detection Kit (Life Technologies, Carlsbad, CA, USA) and analyzed using the StepOne Plus thermocycler (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. In addition, samples were confirmed using selective plating with CT-SMAC (Difco, Detroit, MI, USA) for *E. coli* O157:H7 and XLT-4 (Difco, Detroit, MI, USA) for *Salmonella* spp.

2.3.7 Statistical Analysis

A paired t-test was used for comparison of mean Relative Fluorescent Value (RFV) for post-concentration vs. pre-concentration of filtration methods for each spiking level and hours of enrichment. Significance was determined at p-value level of ≤ 0.05.

2.4 Results

2.4.1 Experimental Design

A schematic of the sample preparation and detection of spiked irrigation water for both pre- and post-concentration samples is detailed in Figure 1. A 25- mL aliquot of the spiked irrigation water was added to 225 mL of selective enrichment broth with supplement which served as the pre-concentrate analyte from the 10-L sample. This method is an example of the standard method determined by the manufacturer (*18*). The MMS gauze or DIF filter cassette served as the post-concentration analyte from the 10-L sample. We developed this method of our sample preparation method we developed using filtration as a concentration device (*22*, *28*). The spiking level selected for *E. coli* O157:H7 MMS concentration were 0 (negative control), 1, 10, 1000 CFU/10L. The spiking level 100 was removed from the detection of *E. coli* O157:H7 MMS concentration due to preliminary work. The spiking levels for *E. coli* O157:H7 DIF concentration and *Salmonella* spp. DIF and MMS concentration were 0 (negative control), 1, 10, and 100 CFU/10L. A total of 3 replicate samples were gathered from each spiking level. The hours of enrichment were 0, 8, 12, and 18. Screening for the target pathogens was done using VIDAS UP® *E. coli* (including H7) and VIDAS UP® *Salmonella* (SPT) and presumptive positive samples were confirmed by RT-PCR and selective media.

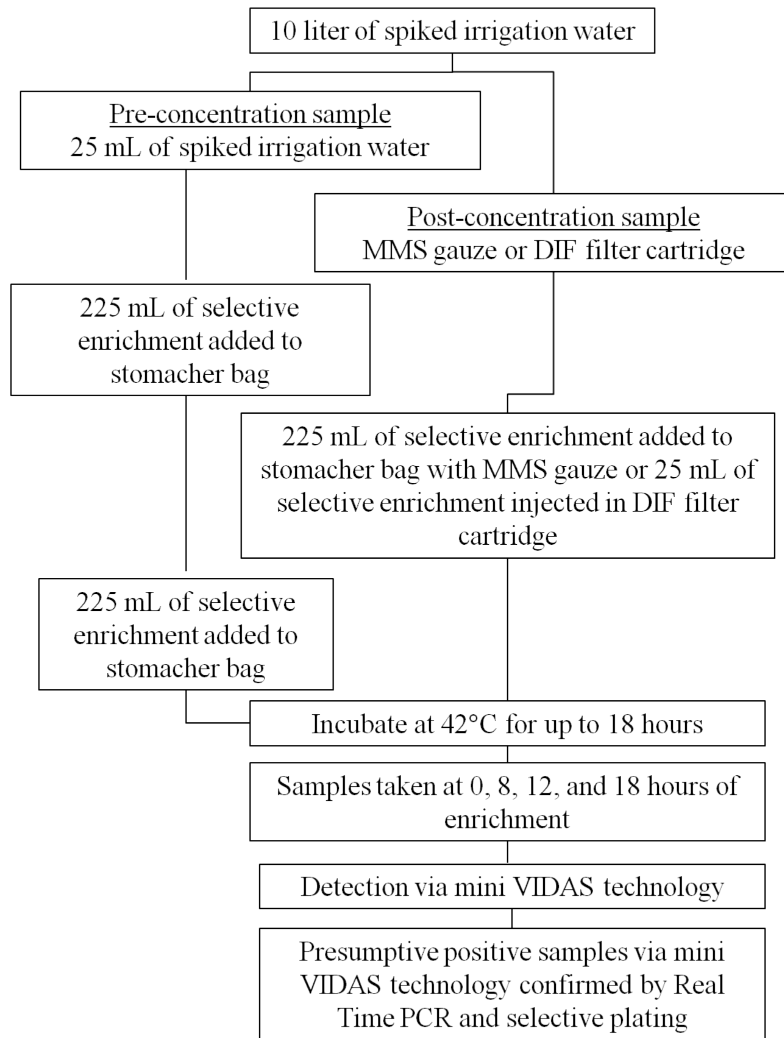

Figure 2. 1 Scheme of pre- and post-concentration sample preparation methods and subsequent detection via mini-VIDAS technology.

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2.4.2 Detection of *E. coli* O157:H7 using VIDAS® Up Technology

Detection using the VIDAS UP® *E. coli* (including H7) assay for MMS concentration is shown in Table 2.1, demonstrating the time to detect *E. coli* O157:H7 in pre- and post-concentration from the spiked irrigation water samples. All pre- MMS concentration replicates were not detected according to mini VIDAS methodology. One negative control post- MMS concentration replicates was detected according to mini VIDAS detection. Observations of post-

and pre- concentrations were not different (*P* = 0.3981). Although typical colonies appeared on the selective media the RT- PCR did not detect the presence of *E. coli* with that replicate. All pre- and post- MMS concentrations replicates for 1 and 10 CFU/10L were not detected according to mini VIDAS detection. There was 1 replicate detected at 8 hours of enrichment and all 3 replicates were detected at 12 and 18 hours of enrichment for spiking level 1000 post- MMS concentration. The difference observed between post- vs. pre- concentration was not different (*P* = 0.0819). There were differences (*P* = 0.00207 and 0.01365) observed for 12 (*P* = 0.00207) and 18 hours (*P* = 0.01365) of enrichment for 1000 CFU/10L.

Detection using the VIDAS UP® *E. coli* (including H7) assay for DIF concentration is shown in Table 2.2, demonstrating the time to detect *E. coli* in pre- and post- concentration from the spiked irrigation water samples. All pre- and post- DIF concentration replicates for the negative control replicates (0 CFU/10L) were not detected according to mini VIDAS detection. All the pre- DIF concentration replicates for 1, 10, and 100 CFU/10L were not detected according to VIDAS technology. One post-DIF concentration replicate was detected at 8 hours of enrichment for 1 and 10 CFU/10L, it was not statistically different (*P* = 0.3981 and 0.41870) and was observed in the post vs. pre comparison. Spiking level 1 CFU/10L was not detected according to VIDAS technology at 12 and 18 hours of enrichment. Detection according to VIDAS technology continued for post- DIF concentration 10 CFU/10L at 12 and 18 hours of enrichment. The difference observed between post- vs. pre- concentration was not different (*P* = 0.34702 and 0.37181). All 3 replicates were detected at 8, 12, and 18 hours of enrichment for spiking level 100 post-DIF concentration. The difference observed between post- vs. pre- concentration was not statistically different (*P* = 0.19189, 0.05453 and 0.05314) at 8, 12, and 18 hours of enrichment respectively for the post vs. pre comparison, respectively.

Table 2. 1 Comparison of VIDAS UP® *Escherichia coli* (including H7) assay using post- MMS concentration and pre- concentration for *Escherichia coli* O157:H7 detection in non-spiked and spiked water sample concentrates.

			No. of samples positive by: Modified Moore Swab			
			Hours of Enrichment			
CFU/10L	Concentration	*n*	0	8	12	18
0	Pre	3	-	-	-	-
	Post	3	-	1 (33%)	1 (33%)	1 (33%)
10	Pre	3	-	-	-	-
	Post	3	-	-	-	-
100	Pre	3	-	-	-	-
	Post	3	-	-	-	-
1000	Pre	3	-	-	-	-
	Post	3	-	1 (33%)	3 (100%)[a]	3 (100%)[a]

[a]Significant dfference observed by paired- t-test ($P < 0.05$)

Table 2. 2 Comparison of VIDAS UP® *Escherichia coli* (including H7) assay using post- DIF concentration and pre- concentration for *Escherichia coli* O157:H7 detection in non-spiked and spiked water sample concentrates.

			No. of samples positive by: Disposible Inline Filter			
			Hours of Enrichment			
CFU/10L	Concentration	*n*	0	8	12	18
0	Pre	3	-	-	-	-
	Post	3	-	-	-	-
1	Pre	3	-	-	-	-
	Post	3	-	1 (33%)	-	-
10	Pre	3	-	-	-	-
	Post	3	-	1 (33%)	1 (33%)	1 (33%)
100	Pre	3	-	-	-	-
	Post	3	-	3 (100%)	3 (100%)	3 (100%)

[a]Significant dfference observed by paired- t-test ($P < 0.05$)

2.4.3 Detection of *Salmonella* spp. using VIDAS® UP Technology

Results from the comparison of mini VIDAS detection post- vs. pre- concentration for the presence of *Salmonella* spp. in spiked irrigation water are detailed in Table 2.3 and 2.4 respectively. Detection using the VIDAS UP® *Salmonella* spp. assay for MMS concentration is shown in Table 2.3, demonstrating the time to detect *Salmonella* spp. in pre- and post- concentration from the spiked irrigation water samples. All pre- and post- MMS concentration replicates for the negative control (0 CFU/10L) and 1 CFU/10L were not detected according to mini VIDAS detection. All of the pre- MMS concentration replicates for 10 CFU/10L and 100 CFU/10L were not detected according to mini VIDAS detection. Spiking level 10 CFU/10L was detected at only 18 hours of enrichment for 2 post- MMS concentration replicates were detected using VIDAS technology. The difference observed between post- vs. pre- concentration was not different (P = 0.16139). Spiking level 100 was detected at 12 hours of enrichment for 2 post- MMS concentration replicates according to VIDAS technology. The difference observed between post- vs. pre- concentration was not different (P = 0.2979). All three replicates at spiking level 100 CFU/10L for post-MMS concentration was detected at 18 hours of enrichment. The difference observed between post- vs. pre- concentration was not statistically different (P = 0.1531).

Detection using the VIDAS UP® *Salmonella* spp. assay for DIF is shown in Table 2.4, demonstrating the time to detect *Salmonella* spp. in pre- and post- concentration from the spiked irrigation water samples. All pre- and post- DIF concentration replicates for the negative control (0 CFU/10L) and 1 CFU/10L were not detected according to mini VIDAS methodology. All 3 replicates were detected at spiking level 10e1 and 100 CFU/10L and were detected post- DIF concentration according to VIDAS technology at 12 and 18 hours of enrichment. The difference

observed between post- vs. pre- concentration was not different (P = 0.12048) at 12 hours of enrichment but was different (P = 0.00467) at 18 hours of enrichment in the post vs. pre comparison. The difference observed between post- vs. pre- concentration was not different for 100 CFU/10L (P = 0.005214 and 0.00064) at 12 and 18 hours.

Table 2. 3 Comparison of VIDAS UP® *Salmonella* (SPT) assay using post- MMS concentration and pre- concentration for *Salmonella* spp. detection in non-spiked and spiked water sample concentrates.

			No. of samples positive by: Modified Moore Swab			
			Hours of Enrichment			
CFU/10L	Concentration	*n*	0	8	12	18
0	Pre	3	-	-	-	-
	Post	3	-	-	-	-
1	Pre	3	-	-	-	-
	Post	3	-	-	-	-
10	Pre	3	-	-	-	-
	Post	3	-	-	-	2 (66%)
100	Pre	3	-	-	-	-
	Post	3	-	-	2 (66%)	3 (100%)

[a]Significant dfference observed by paired- t-test (P < 0.05)

Table 2. 4 Comparison of VIDAS UP® *Salmonella* (SPT) assay using post- DIF concentration and pre- concentration for *Salmonella* spp. detection in non-spiked and spiked water sample concentrates.

			No. of samples positive by: Disposible Inline Filter			
			Hours of Enrichment			
CFU/10L	Concentration	*n*	0	8	12	18
0	Pre	3	-	-	-	-
	Post	3	-	-	-	-
1	Pre	3	-	-	-	-
	Post	3	-	-	-	-
10	Pre	3	-	-	-	-
	Post	3	-	-	3 (100%)	3 (100%)[a]
100	Pre	3	-	-	-	-
	Post	3	-	-	3 (100%)[a]	3 (100%)[a]

[a]Significant dfference observed by paired- t-test ($P < 0.05$)

2.5 Discussion

With the increase in demand for fresh ready-to-eat products including minimally processed fruits and vegetables, there is a risk of contamination with foodborne pathogens (*37*). Detection of foodborne pathogens such as *Salmonella* spp. and *Escherichia coli* O157:H7 in fresh produce is impractical due to small sample size, variability of contamination, and low sensitivity of current methods (*22*, *93*, *126*). As an alternative to traditional microbiological analysis, the food industry is constantly exploring rapid and sensitive techniques such as the mini VIDAS UP, a molecular based method with high- throughput and rapid screening (*19*, *37*, *88*, *125*, *155*, *163*). In our study we used the phage technology base assay VIDAS® UP for both *E. coli* O157:H7 and *Salmonella* spp. for detection while incorporating a single stream sample preparation method of concentration with filtration method. One-step enrichment increased the detection of pathogens in spiked irrigation water samples.

Previous studies have compared the detection of mini-VIDAS platform and kits to both conventional and molecular methods for food products and milk production (*19*, *37*, *88*, *125*, *163*). Elizaquível et al. (2009) compared the detection of real-time polymerase chain reaction based method (RTi-PCR) to mini – VIDAS SLM kit (bioMéreiux®) on naturally contaminated food products. In that study, the RTi-PCR outperformed the VIDAS as a better screening method for *Salmonella* detection in food products. However, after re-evaluating the RTi-PCR method, the researchers found that analysis can be thoroughly completed within 24 hours which meets the current criteria for *Salmonella* spp. require absence in specific amounts. The analysis begins after direct homogenization along with simultaneous incubation to eliminate false-negative results. Zadernowska et al. (2014) ascertained that previous studies have demonstrated the high sensitivity of the SLM application but that false positives may result from assays that are overloaded with accompanying microflora. In the study VIDA UP and Fluorescence In Situ Hybridization (FISH) was compared as an alternative method for detection of *Salmonella* enterica serovars in meat. They found that both methods were comparable in substantial reduction of waiting time for results, highly sensitive, and specific but mini-VIDAS was found to be less complicated for less experienced lab workers. Having easy to use rapid detection devices with simple sample preparation is key to helping maintain food safety standards in the processing area. The bioMéreiux Company has transitioned from the Enzyme Immunoassay (EIA) base screening technique *Salmonella* (SLM) assay to the *Salmonella* Phage Technology (SPT) with results produced in less than 19 hours. The SPT application uses recombinant phage proteins specific to *Salmonella* spp. labeled with alkaline phosphatase instead of the antigen-antibody reaction which occurs with the SLM assay (*163*).

Walker et al. (2001) compared *Salmonella* detection using the VIDAS SLM assay and recommended the reference sampling method and Moore swab sampling method in bulk milk and in-line milk filters. In the comparison analysis the overall agreement between the two methods was 91.33%. The biggest difference between these methods was that larger volumes of milk (1L) could be sampled with the Moore swab than the standard culture method which recommends 25 mL of milk per sample. In our study the standard method was also evaluated as the pre-concentration sample in which a 25-mL of spiked irrigation water sample was taken for enrichment. Using a small size for the pre-concentration sample reduced the ability to detect the pathogen therefore concentrating the bacteria using DIF or MMS increased the detectability in large (10L) volume samples. In agreement with our study, Walker et al. (2001) found a greater likelihood of detecting *Salmonella* using the Moore swab sampling method along with proper enrichment broth. The authors also mentioned that the use of selective media could have also prevented overgrowth of contamination organisms. In this study they observed that using the Moore swab was slightly more sensitive than the manufacturers' method yet no significant difference was observed. The same was observed with our study, there were instances where 2 out of 3 post- concentration replicates were positives yet, no significant difference was observed between post- vs. pre- concentration.

Walker et al. (2001) also evaluated inline filters and found a 95.57% agreement with the conventional culture method (*155*). They explored this form of filtration because of claims of higher sensitivity for assessing the presence of *Salmonella* in milk from dairies rather than direct sampling. They also observed that the inline filters sample a larger amount of milk samples along with the same rationale of using Moore-swab sampling.

2.6 Conclusion

Overall, in our study we developed a concentration method via MMS and DIF coupled with detection using the mini-VIDAS technology to sensitively and specifically detect *Salmonella* spp. and *E. coli* O157:H7 in irrigation water. Using these methods we were able to detection the pathogens within 18 hours of enrichment in comparison to standard sampling methods. The concentration protocol developed employed shortened time-to-detect and improved sensitivity compared to previously established methodology.

CHAPTER 3

A Comparison of Methods to Serotype *Salmonella enterica* Isolates from Food and Agricultural Environments

3.1 Summary

Serotyping of *S. enterica* isolates is essential for developing risk management strategies for food production and implementing corrective actions during foodborne illness outbreaks. Traditionally, serotyping of *S. enterica* is based on the method of immunological-based agglutination reactions. This procedure is laborious, often difficult to interpret, and not amenable to high throughput workflows. The objective of this study was to compare slide agglutination serotyping to three molecular typing strategies used to characterize *S. enterica*: riboprinting, pulsed-field gel electrophoresis (PFGE), and the Luminex xMAP *Salmonella* Serotyping Assay. A diverse panel of food, outbreak, and agricultural source *S. enterica* isolates (n = 145) were evaluated by riboprinting using *Pvu*II restriction enzymes, by PFGE using *XbaI*, and by xMAP assay. The outputs from each molecular method were compared to slide agglutination serotyping output. Strong agreement of serotyping results were observed for food- and outbreak-related *S. enterica* isolates when comparing molecular typing strategies to slide agglutination serotyping. Automated riboprinting and PFGE were in strong agreement with outbreak serotypes *S.* Anatum, *S.* Newport, and *S.* Typhimurium. Serotypes not frequently observed in clinical/veterinary settings were often problematic to serotype with molecular methods when obtained from the environment. Slide agglutination serotyping identified several isolates as *S.* Senftenberg, but SSA could not independently identify the isolate due the inability

to detected H:t allele of Senftenberg. Overall, the majority of *S.* enterica serotypes can be effectively typed using molecular serotyping methods.

3.2 Introduction

Infections with non-typhoidal *Salmonella* currently account for 35% of hospitalizations and 28% of the mortalities related to foodborne disease in the United States (*128*). Globally, 93.8 million cases of *Salmonella*-linked gastroenteritis are estimated to occur annually and result in 155,000 deaths, with the majority of cases stemming from food contamination (*91*). Over 2,600 *S.* enterica serotypes are recognized, although most are capable of causing disease in human, only a small portion of these are frequently associated with veterinary or clinical disease (*70*). This necessitates highly specific diagnostics to characterize and differentiate problem *S.* enterica serotypes from those lacking agricultural or clinical relevance. The gold standard of *S.* enterica serotype differentiation is the Kaufmann-White-Le Minor classification (KWL) scheme which utilizes antisera agglutination assays to identify serotype-specific polymorphisms in the somatic (O), flagellar (H), and capsular (Vi) antigens (*121*, *157*). However, misinterpretation of these assays can result from weak or nonspecific agglutination reactions, loss of antigen expression, manufacturer and lot-to-lot variability of antisera, and technician error (*121*, *129*, *133*).

More recently, DNA-based serotyping or so called “molecular serotyping” methods were developed to be user friendly, achieve more rapid results, and add fingerprinting capacity for *S.* enterica characterization (*164*). Among the most widely used of these methods in the food industry are pulsed-field gel electrophoresis (PFGE), RiboPrinting (DuPont Qualicon, Wilmington, DE, USA), and the xMAP *Salmonella* Serotyping Assay (Luminex, Austin, TX) (*133*, *157*). While PFGE and Riboprinting were intended for subtyping applications, both have

demonstrated the ability to identify serotypes. PFGE is reportedly able to correctly serotype between 75% to 99% of *Salmonella* isolates whereas the serotyping accuracy of Riboprinting ranges from approximately 39% to 100% of *Salmonella* isolates (*133*). The functionality of both these methods are facilitated by evolvable databases of reference fingerprints, including Centers for Disease Control and Prevention's Pulse Net Network and the DuPont Identification Library as part of the RiboPrinter System (*130*, *141*). Initially developed by CDC researchers, and commercialized by Luminex, the xMAP *Salmonella* assay is specifically designed for classification of the most clinically encountered *S.* enterica serotypes. This assay unifies PCR amplification of the O and H antigen encoding genes as well as a serotype specific markers with a microsphere-based liquid array system for amplicon detection (*36*, *43*, *95*). The xMAP ® *Salmonella* assay reportedly characterizes 90% of most commonly encountered *Salmonella* serotypes in clinical settings, and is used by numerous state public health laboratories and CDC laboratories (*43*, *95*). However, for all molecular serotyping platforms, serotyping efficacy has not been validated for full complements of *S.* enterica serovars, particularly isolates of environmental origin (*40*, *68*, *161*). Thus additional testing with a greater breadth of diverse isolates is required to validate these methods.

In the present study, antisera agglutination assays (KWL scheme), PFGE, Riboprinting, and the xMAP *Salmonella* serotyping assay were utilized to define the serotypes of 145 *S.* enterica isolates collected from agricultural and food production environments. Ninety-three of these isolates were collected from Central Florida surface water sites and serotypically were highly diverse (*94*). Thirty-three isolates were obtained from an outbreak investigation associated with fresh produce and 19 isolates from a food production facility. Strong agreement of serotyping results were observed for food- and outbreak-related *S.* enterica isolates however

agricultural isolates proved more problematic with frequent discrepancies between molecular serotyping methods. The majority of *S.* enterica serotypes can be effectively typed using molecular serotyping methods.

3.3 Materials and Methods

3.3.1 Collection of isolates and culturing conditions

S. enterica isolates (n = 145) were collected from Central Florida surface waters (n = 93), the site of an outbreak associated with fresh produce (n = 33), and a food production facility (n = 19). The isolation and collection of surface water isolates was performed as described by McEgan et al. (*94*). *S.* enterica isolates collected in association with the fresh produce outbreak were obtained using several drag swabbing methods in parallel. Three types of drag swabs, conventional gauze, paint rollers, and tampons were fixed to 1 meter of cotton string, sterilized, then aseptically pre-moistened with 400 g of sterile canned evaporated milk prior to sampling inside Whirl-Pak bags. The swabs were then dragged in a "U" or "W" pattern across the sampling sites. *S.* enterica isolates from the production facility were also collected. Initial screening of both fresh produce outbreak and production facility isolates for *S.* enterica presence/absence was conducted after a one step enrichment using the BAX Automated System following manufacturer's instructions and according to AOAC 2003.09 BAX (*5*). Enrichments considered presumptive positive by PCR were subjected to culture-based isolation procedures following procedures delineated by the U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) with slight modifications. Namely, selective plating onto xylose lysine desoxycholate agar (XLD), bismuth sulfite agar (BS), or hektoen enteric agar (HE) was conducted directly from the one-step enrichment broth, followed by biochemical

confirmation using TSI/LIA and API 20E and traditional serotyping (*147*). All isolates were stored in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 20% glycerol at -80°C.

3.3.2 Serotyping via slide agglutination

Traditional serotyping of *S.* enterica isolates using antisera-based agglutination assays was performed by the National Veterinary Laboratory Service (Ames, IA, USA) the Colorado Department of Public Health and Environment (Denver, CO, USA) or Wyoming Public Health Laboratory (Cheyenne, WY, USA) according to standard KWL practices (*70*).

3.3.3 Pulsed-field gel electrophoresis

PFGE was performed in accordance with CDC Pulse Net protocols using *XbaI* restriction for all isolates (*152*). The Pulse Net universal standard strain *Salmonella* enterica Braenderup H9812 was used as the reference marker. Gel images were captured using a Gel Doc XR Imaging System (Bio-Rad, Hercules, CA, USA) and images were analyzed using BioNumerics™ software ver. 6.6 (Applied Maths, Austin, TX). Relatedness of each PFGE pattern was assessed using the unweighted pair group method with arithmetic averages and 1.5% band position tolerances and dice coefficients had 2.0% optimization values. The PFGE patterns and PFGE-predicted serotypes of isolates collected from Central Florida surface waters isolates were described previously (*94*).

3.3.4 Riboprinting

Ribosomal DNA from each isolate was profiled using the RiboPrinter Microbial Characterization System (DuPont Qualicon) in accordance with the manufacturer's instructions. Briefly, single colonies from overnight tryptic soy agar cultures were picked, suspended in sample buffer, and heat-treated before being placed in the automated RiboPrinter Microbial Characterization System. Restriction was then performed with *PvuII*. In this system, the restricted DNA is separated by gel electrophoresis and subsequently transferred to a nylon membrane for Southern blot analyses using DNA probes complementary to ribosomal sequences, yielding a Riboprint pattern. The Riboprint patterns of the isolates were matched to reference patterns was using the DuPont RiboExplorer Software (Ver. 2.2.0232.0).

3.3.5 Luminex xMAP *Salmonella* serotyping assay

The Luminex xMAP *Salmonella* serotyping assay was performed in accordance with the manufacturer's instructions. Genomic DNA was extracted from *S.* enterica isolates grown on tryptic soy agar by suspending a single colony into 20 µl of InstaGene matrix (Bio-Rad) and mixed. Samples were next heated to 56°C for 10 min, allowed to cool, suspended in 100 µl of H2O, and debris removed by centrifugation. Two hundred nanograms of genomic DNA was used for PCR amplification of O antigen, H antigen, AT group gene targets. Each 25-µl PCR reaction contained 12.5 µl of Qiagen HotStar TaqMaster Mix (Qiagen, Valencia, CA), 2.5 µl of either the O antigen, H antigen, or AT group biotinylated primer pool, template and H2O to 25 µl. Thermocycling conditions were 95°C for 15 min; 30 cycles of 94°C for 30 sec, 48°C for 90 sec, 72°C for 90 sec; and a final extension at 72°C for 15 min. Five microliters of each PCR product was then added to 45 µl of 1X O antigen, H antigen, or AT group microspheres prepared

in 1X Assay Buffer (Luminex) for hybridization. Hybridization was achieved by heating samples to 95°C for five minutes and then decreasing the temperature to 52°C and incubating for 30 min. Streptavidin-R-phycoerythrin reporter (50.4 µg) was added and mixed with each reaction (per antigen gene target)immediately prior to analyses using the Bio-Plex 200 System operating using Bio-Plex Manager software (version 6.0.0.617). Detection of specific amplicons was interpreted according to the median fluorescence intensity (MFI) score assigned for each sample by the Bio-Plex system and any reading from an antigen with an MFI value greater than 1000 MFI, a signal-to-noise (S/N) ratio of 6 or greater, or both is considered positive. Correlation of the detected serogroup or AT group specific amplicons to serotypes was achieved using the Luminex *Salmonella* Analysis Tool which follows the presumptive genetic basis of the KWL scheme.

3.4 Results

3.4.1 Serotyping via slide agglutination

Conventional serotyping via slide agglutination allowed for the characterization of all 145 *S.* enterica isolates (93 from Central Florida surface water sites, 33 from an outbreak investigation associated with fresh produce, and 19 from a food production facility), (Table 3.1). A diverse panel of serovars was represented among these isolates (31 serovars from the Central Florida surface waters, 5 serovars from the outbreak investigation associated with fresh produce and 5 serovars from the food production facility, (Table 3.1).

Serovars listed from the CDC's 20 most frequently reported serotypes for laboratory confirmed human Salmonella infections (*150*) were represented in our isolates, (Appendix 1.7 – 1.9). Eight serovars from Central Florida surface water isolates (Anatum, Bareilly, Branderup,

Muenchen, Newport, Paratyphi B var. L (+) tartrate +, Saintpaul, and Typhimurium), 3 serovars from fresh produce outbreak isolates (Anatum, Newport, and Typhimurium) and one serovar from the food production facility isolates (Montevideo).

Table 3. 1 Overall summary of *Salmonella enterica* isolates used in the study.

Source	No. of conventional typed isolates	No. of serovars	No. of typed isolates		
			PFGE	Automated RiboPrinter ®	xMAP
Central Florida Surface Waters	93	31	87 (94%)	77 (83%)	54 (50%)
Food Production Facility	19	5	18 (95%)	17 (89%)	4 (21%)
Fresh Produce Outbreak	33	5	31 (94%)	32 (97%)	31 (94%)
Total	145				

3.4.2 Automated Riboprinter

The automated riboprinter predicted serotypes for 77 of 93 (83%) Central Florida surface water isolates, 17 of 19 (89%) food production facility isolates and 32 of 33 (97%) fresh produce outbreak isolates (Table 3.1). Among these, 43% (40 of 93) of Central Florida surface water isolates, 74% (14 of 19) food production facility isolates, and 94% (31 of 33) fresh produce outbreak isolates characterizations correlated to conventional serotyping data.

By comparison to conventional serotyping the Florida surface water isolates serovar incorrectly identified by Riboprinting were: III_44:z4,z32:- (2 isolates), IV_50:z4,z23:- (1isolate), Rough_O:d:1,7 (2 isolates), Rough O: y:1,5 (2 isolates), 6,8:d:- (1 isolate), Baildon (2 isolates), Bareilly(1 isolate), Braenderup (5 isolates), Florida (1 isolate), Gaminara (12 isolates), Muenchen (1 isolate), Rubislaw (7 isolates) (Table 3.2). The food production facility isolates serovar that were incorrectly identified were: Kouka (1 isolate), and R:e,n,x (2 isolates) (Table 3.2). One fresh produce outbreak isolate serovar was incorrectly identified, Bareilly (Table 3.2).

Often the Automated RiboPrinter® cannot match a ribotype pattern of an isolates to the pattern in its database. In that case, those isolates were labeled as "could not be identified with confidence." The following Florida surface waters isolates serovar (determined by conventional serotyping) were not identified: III_17:z10:e,n,x,z15 (1 isolate), IV Rough O:z4,z24:- (2 isolates), IV Rough O:z4,z23:- (1 isolate), IV_50:z4,z23:-(1 isolate), 6,8:d:- (1 isolate), 50:z4,z23:-(1 isolate), Florida (1 isolate), Georgia (1 isolate), Give (1 isolate), Ituri (2 isolates), Litchfield (1 isolate), Norwich (2 isolates), Saintpaul (1 isolate), (Table 3.2). Three food production facility isolates serovar were not identified: L:e,n,x (1 isolate) and Montevideo (1 isolate), (Table 3.2). Only one fresh produce outbreak isolates serovar was not identified, which was initially characterized by conventional serotyping as *S.* Newport (Table 3.2).

3.4.3 Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis predicted serotypes for 87 of 93 (94%) Central Florida surface water isolates, 18 of 19 (95%) food production facility isolates and 31 of 33 (94%) fresh produce outbreak isolates (Table 3.1). Among these, isolates with serovars congruent with traditional serotyping were: 66 of 93 (71%) of Central Florida surface water, 14 of 19 (74%) food production facility, and 31 of 33 (94%) fresh produce outbreak (Table 3.2).

The Florida surface water isolates serovars incorrectly characterized compared to conventional serotyping were: IV_50:z4,z23:- (1 isolate), Rough_O:d:1,7 (2 isolates), Rough O: y:1,5 (2 isolates), IV Rough O:z4,z24:- (2 isolates), 50:z4,z23:- (1 isolate), Gaminara (6 isolates), Muenchen (1 isolate), Paratyphi B var. L-tartrate+ (1 isolate), (Table 3.2). The food production isolates serovar incorrectly identified were: L:e,n,x (1 isolate), Kouka (1 isolate), and R:e,n,x (2 isolate), (Table 3.2). There were no fresh produce isolates identified incorrectly (Table 3.2).

There were some isolates in which the Pulse Net database could not match the pattern to known serotypes. Thus, the serotype identification of these isolates was defined as “could not be identified with confidence.” The following Florida surface waters isolates serovar were not identified (Table 3.2): III_17:z10:e,n,x,z15 (1 isolate), III_44:z4,z32:- (2 isolates), IV Rough O:z4,z23:- (1 isolate), 6,8:d:- (2 isolates), Florida (2 isolates), Gaminara (1 isolate), Georgia (1 isolate), Give (1 isolate). Only one isolate from the food production facility was serotyped as Montevideo using the conventional method and not identified using PFGE, (Table 3.2). There were two isolates from the fresh produce outbreak serotyped as Bareilly and another as Newport with conventional methods and not identified using PFGE (Table 3.2).

3.4.5 Luminex® (xMAP) *Salmonella* Serotyping Assay

The Luminex ® *Salmonella* Serotyping assay produced an output for 54 of 93 (50%) Central Florida surface water isolates, 4 of 19 (21%) food production facility isolates and 31 of 33 (94%) fresh produce outbreak isolates (Table 3.1). Isolates characterizations that matched conventional serotyping were (Table 2): 37 of 93 (40%) of Central Florida surface water, 14 of 19 (74%) food production facility, and 31 of 33 (94%) fresh produce outbreak (Table 3.2).

It was frequently observed that a specific serotype could not be identified as demonstrated by isolates that were characterized as Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum; Baildon/Lomalina/ ll[1],9,12:a:e,n,x; Virgina/Muenchen; Rubislaw/Kibusi; and Newport/Bardo or Larochelle (Appendix 1.3 – 1.5). One group of isolates was identified incorrectly when compared to the conventional method. The Florida surface water isolates serovar incorrectly identified were: IV_50:z4,z23:- (1 isolate), Anatum (3 isolates), Braenderup (3 isolates), Gaminara (1 isolate), Georgia (1 isolate), Hartford (1 isolate), Ituri (1 isolate),

Miami (2 isolates), Muenchen (3 isolates), Saintpaul (1 isolate), (Table 3.2). Only two serovars were incorrectly identified for the food production facility: Kouka (1 isolate), R:e,n,x (2 isolates), (Table 3.2). None of fresh produce outbreak serovars were incorrectly identified (Table 3.2).

The Luminex ® *Salmonella* serotyping assay was developed to characterize 90% of most commonly encountered *Salmonella* serotypes in clinical settings, and is use in numerous state public health laboratories and CDC laboratories (*43*, *95*). In our study, we found that identification of the isolates obtained from agricultural sources was problematic and those isolates were labeled as "could not be identified with confidence." The following Florida surface waters isolates serovar were not identified with SSA (Table 3.2): III_17:z10:e,n,x,z15 (1 isolate), III_44:z4,z32:- (2 isolates), Rough_O:d:1,7 (2 isolates), Rough O: y:1,5 (2 isolates), IV Rough O:z4,z24:- (2 isolates), IV Rough O:z4,z23:- (1 isolate), IV_50:z4,z23:- (1 isolate), 6,8:d:- (2 isolates), 50:z4,z23:- (1 isolate), Bareilly (1 isolate), Braenderup (1 isolate), Florida (2 isolates), Gaminara (13 isolates), Give (1 isolate), Inverness (1 isolate), Litchfield (1 isolate), Norwich (2 isolates), Oranienburg (1 isolate), Paratyphi B var. L-tartrate+ (2 isolates) Only two serovars from the food production facility serotypes were not identified using the xMAP assay: L: e,n,x (1 isolate), Senftenberg (14 isolates), (Table 3.2). There were also two serovars from the fresh produce outbreak isolates serotypes not identified using the xMAP assay (Table 3.2): Javiana (1 isolate) and Newport (1 isolate).

Table 3. 2 Comparison of DNA-based serotyping method used to predict the serovars of isolates from Central Florida surface waters, food production facility, and fresh produce outbreak.

	DNA-based Serotyping Method	No. of isolates (n=)	No. of isolates of which the serovar was identified correctly (%)	Incorrectly identified *S. enterica* serovars (no.)	*S. enterica* serovars not identified (no.)
Central Florida Surface Waters		93			
	PFGE		66 (71%)	IV_50:z4,z23:- (1), Rough_O:d:1,7 (2), Rough O: y:1,5 (2), IV Rough O:z4,z24:- (2), 50:z4,z23:- (1), Gaminara (6), Muenchen (1), Paratyphi B var. L-tartrate+ (1)	III_17:z10:e,n,x,z15 (1), III_44:z4,z32:- (2), IV Rough O:z4,z23:- (1), 6,8:d:- (2), Florida (2), Gaminara (1), Georgia (1), Give (1)
	Automated RiboPrinter ®		40 (43%)	III_44:z4,z32:- (2), IV_50:z4,z23:- (1), Rough_O:d:1,7 (2), Rough O: y:1,5 (2), 6,8:d:- (1), Baildon (2), Bareilly(1), Braenderup (5), Florida (1), Gaminara (12), Muenchen (1), Rubislaw (7)	III_17:z10:e,n,x,z15 (1), IV Rough O:z4,z24:- (2), IV Rough O:z4,z23:-(1), IV_50:z4,z23:-(1), 6,8:d:- (1), 50:z4,z23:-(1), Florida (1), Georgia (1), Give (1), Ituri (2), Litchfield (1), Norwich (2), Saintpaul (1)
	Luminex® xMAP *Salmonella* serotyping assay		37 (40%)	IV_50:z4,z23:- (1), Anatum (3), Braenderup (3), Gaminara (1), Georgia (1), Hartford (1), Ituri (1), Miami (2), Muenchen (3), Saintpaul (1)	III_17:z10:e,n,x,z15 (1), III_44:z4,z32:- (2), Rough_O:d:1,7 (2), Rough O: y:1,5 (2), IV Rough O:z4,z24:- (2), IV Rough O:z4,z23:- (1), IV_50:z4,z23:- (1), 6,8:d:- (2), 50:z4,z23:- (1), Bareilly (1), Braenderup (1), Florida (2), Gaminara (13), Give (1), Inverness (1), Litchfield (1), Norwich (2), Oranienburg (1), Paratyphi B var. L-tartrate+ (2)
Food Production Facility		19			
	PFGE		14 (74%)	L: e,n,x (1), Kouka (1), R:e.n.x (2)	Montevideo (1)
	Automated RiboPrinter ®		14 (74%)	Kouka (1), R:e,n,x (2)	L: e,n,x (1), Montevideo (1)
	Luminex® xMAP *Salmonella* serotyping assay		1 (5%)	Kouka (1), R:e,n,x (2)	L: e,n,x (1), Senftenberg (14)
Fresh Produce Outbreak		33			
	PFGE		31 (94%)		Bareilly (1), Newport (1)
	Automated RiboPrinter ®		31 (94%)	Bareilly (1)	Newport (1)
	Luminex® xMAP *Salmonella* serotyping assay		31 (94%)		Javiana (1), Newport (1)

3.4.6 Cluster analysis of patterns produced by PFGE and Automated RiboPrinter ®

Cluster analyses of PFGE banding patterns from all isolates (Central Florida surface water, food production facility, and fresh produce outbreak) were performed to evaluate differences and similarities among patterns (Appendix 1.6 – 1.9) and also for Automated RiboPriner® banding patterns (Appendix 1.10 – 1.13). All isolates of *Salmonella* enterica serotype Senftenberg isolates from the food production facility clustered as identical by PFGE and Automated RiboPrinter ® (Figures 3.1 and 3.2). The same was observed for serovars Typhimurium (Figures 3.3 and 3.4) from the isolate obtained from a fresh produce outbreak by both PFGE and Automated RibPrinter®. Many of the isolates serotyped as Anatum from Central Florida surface water and the fresh produce outbreak isolates clustered as identical as well by PFGE and Automated Riboprinter® (Figures 3.5 and 3.6). Newport isolates generated various genotypic clusters by both PFGE and Automated RibPrinter® (Figures 3.7 and 3.8).

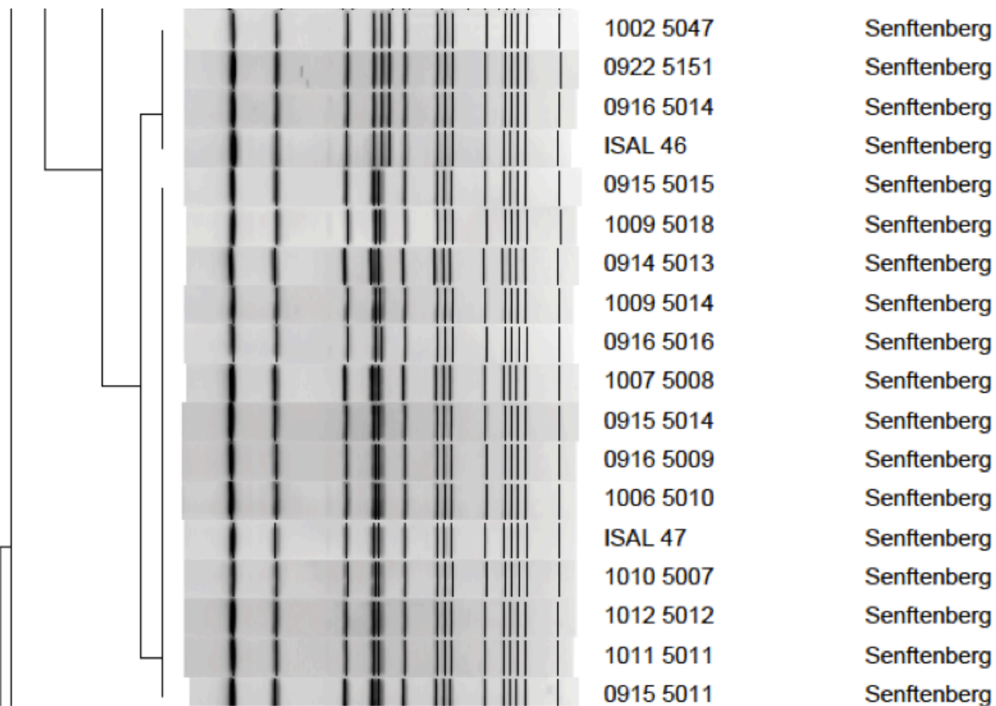

Figure 3. 1 Dendrogram of *Salmonella enterica* serovar Senftenberg recovered isolates from food processing facility cluster, the largest cluster of isolates >80% similar to each other determined by PFGE –Xbal fingerprinting.

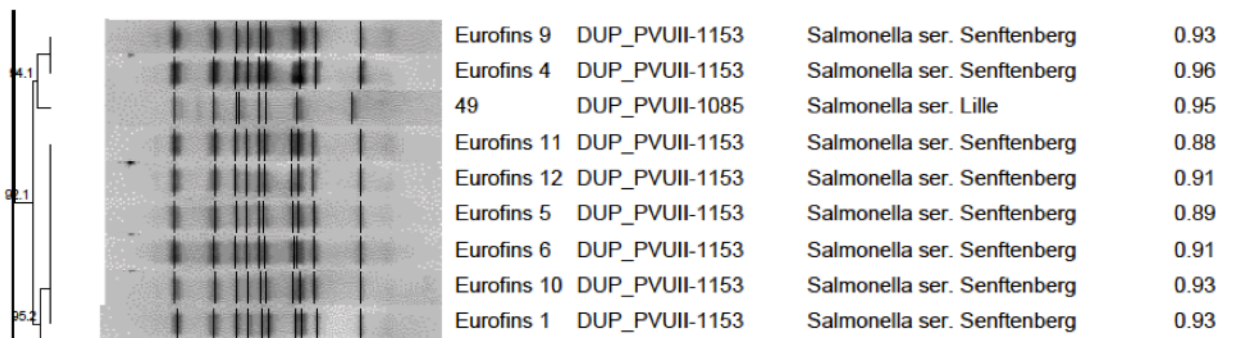

Figure 3. 2 Dendrogram of *Salmonella enterica* serovar Senftenberg recovered isolates from food processing facility cluster, the largest cluster of isolates >80% similar to each other determined by Automated RiboPrinter® –PvuII.

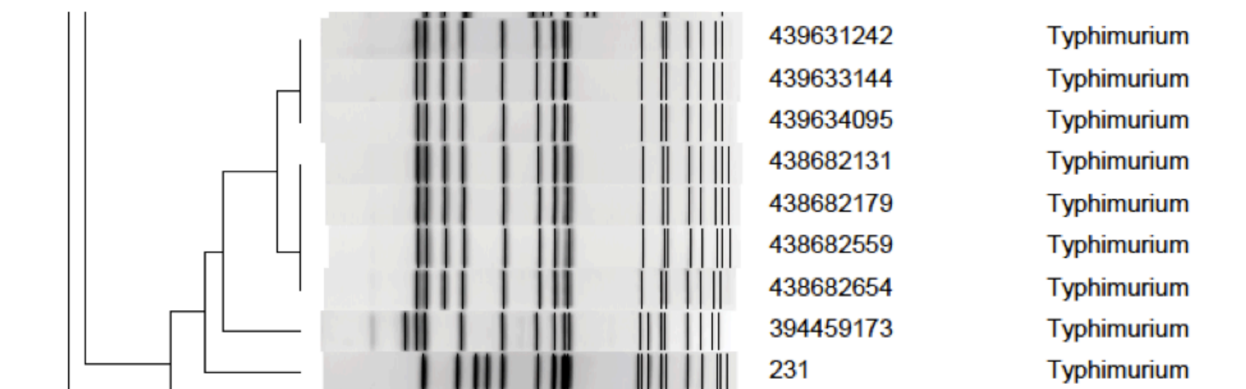

Figure 3. 3 Dendrogram of *Salmonella enterica* serovar Typhimurium recovered isolates from fresh produce outbreak cluster, the largest cluster of isolates >80% similar to each other determined by PFGE –Xbal fingerprinting.

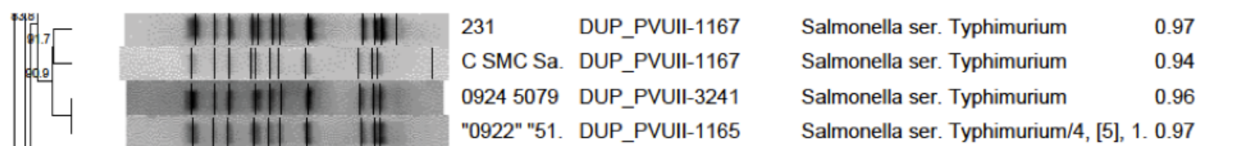

Figure 3. 4 Dendrogram of *Salmonella enterica* serovar Typhimurium recovered isolates from fresh produce outbreak and Central Florida surface waters cluster, the largest cluster of isolates >80% similar to each other determined by Automated RiboPrinter® –PvuII.

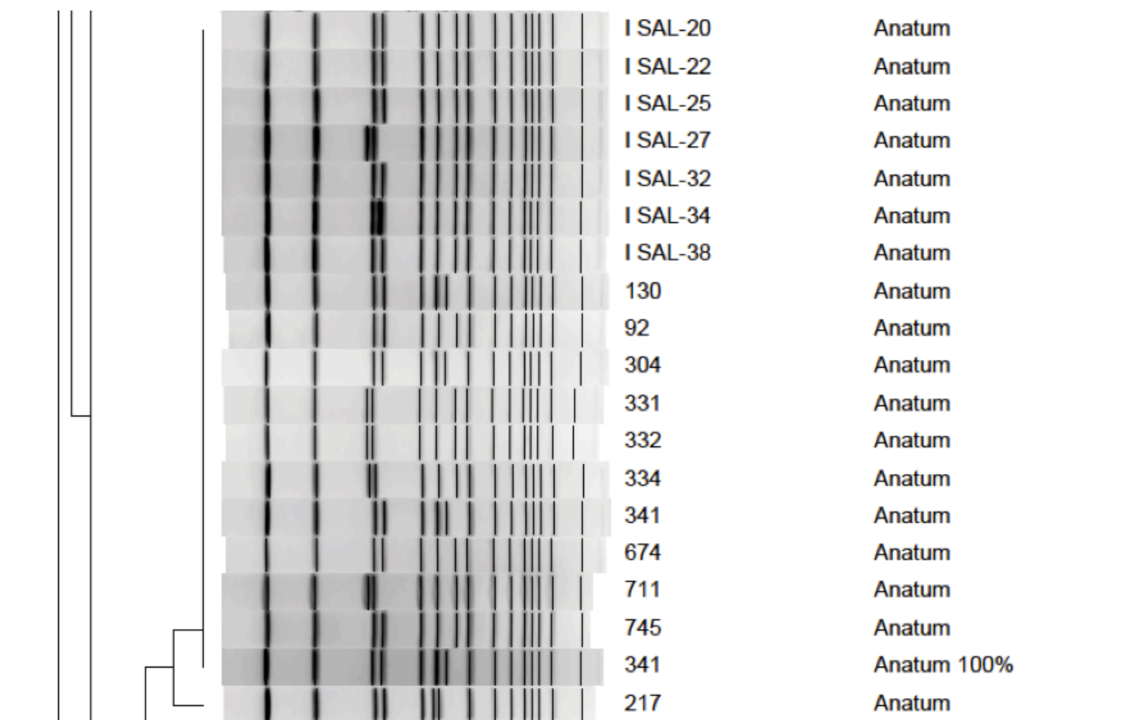

Figure 3. 5 Dendrogram of *Salmonella enterica* serovar Anatum recovered isolates from fresh produce outbreak and Central Florida surface water cluster, the largest cluster of isolates >80% similar to each other determined by PFGE –Xbal fingerprinting.

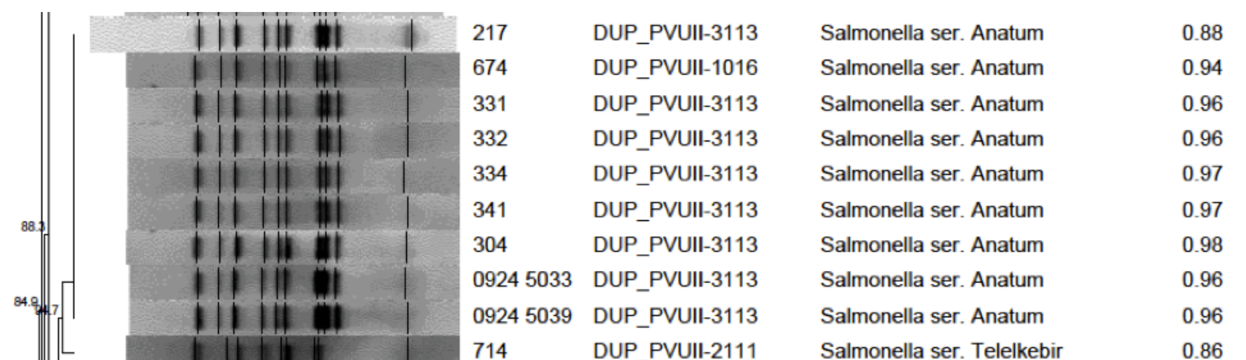

Figure 3. 6 Dendrogram of *Salmonella enterica* serovar Anatum recovered isolates from fresh produce outbreak and Central Florida surface water cluster, the largest cluster of isolates >80% similar to each other determined by Automated RiboPrinter® –PvuII.

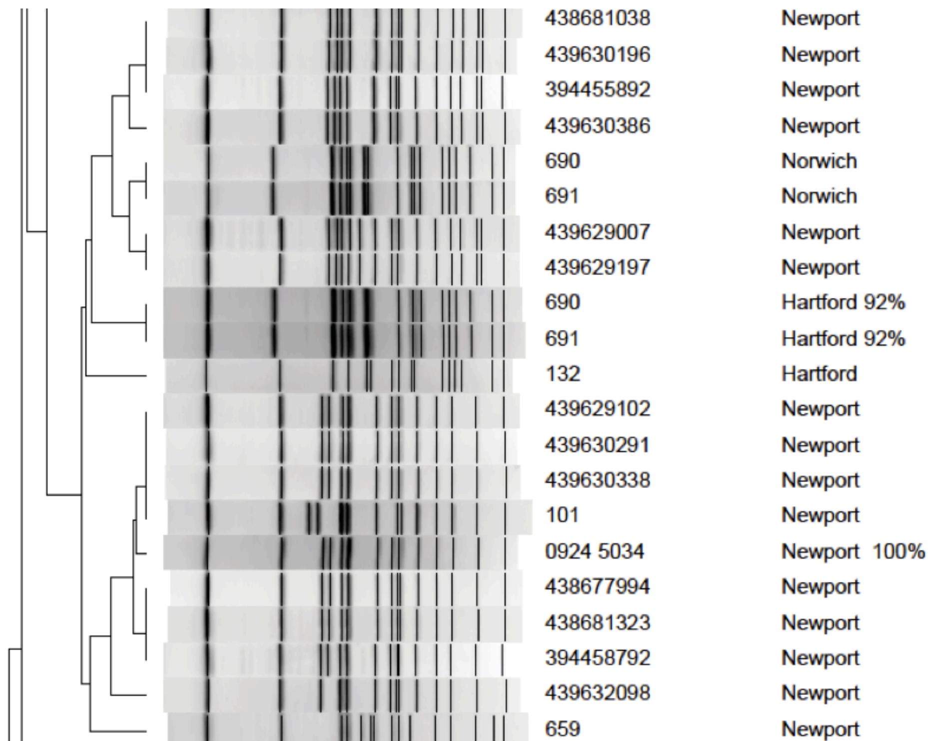

Figure 3. 7 Dendrogram of *Salmonella enterica* serovar Newport recovered isolates from fresh produce outbreak cluster, the largest cluster of isolates >80% similar to each other determined by PFGE –Xbal fingerprinting.

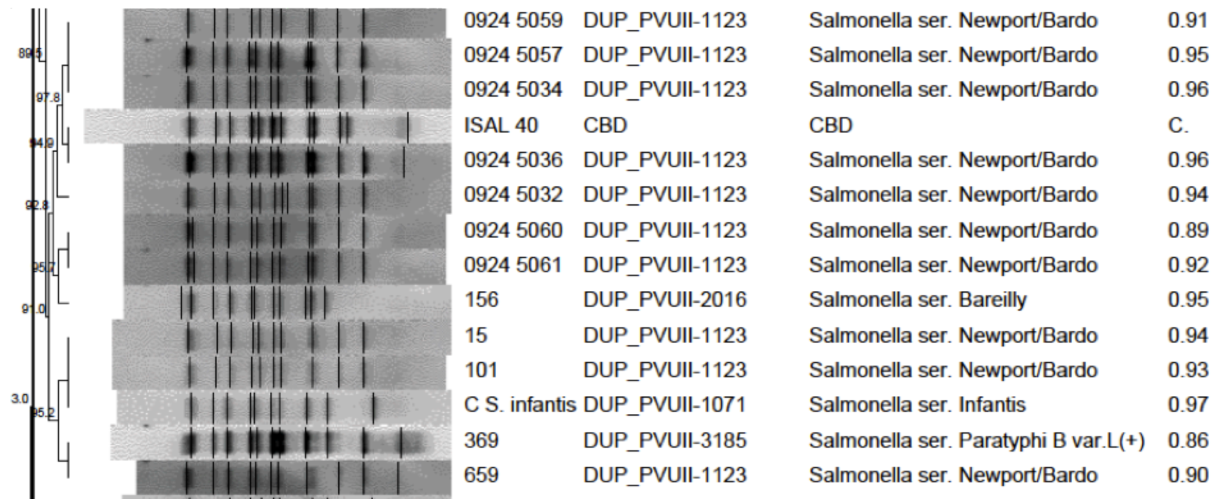

Figure 3. 8 Dendrogram of *Salmonella enterica* serovar Newport recovered isolates from fresh produce outbreak and Central Florida surface waters cluster, the largest cluster of isolates >80% similar to each other determined by Automated RiboPrinter® –PvuII.

3.5 Discussion

The White-Kauffmann-Le minor scheme used in conventional serotyping has identified over 2,600 *Salmonella* serovars based on the classification of 46 different somatic serogroups and 114 different flagellar antigens in several combinations (*70*, *133*). In this study, there were 31 serovars identified for the Florida surface water isolates, 5 serovars for the food production facility and fresh produce outbreak isolates. Serovars Typhimurium and Newport were listed among those for both the surface water and fresh produce outbreak isolates of which are commonly associated with a wide variety of foods and environmental settings (*32*, *42*, *66*, *71*, *90*, *116*). Several serovars frequently associated with animal derived food commodities were identified, such as Braenderup, Javiana, and Saintpaul, were also observed in both surface water and fresh product outbreak isolates. Serovars associated with plant and food commodities, including Javiana, Litchfield, Muenchen, Saintpaul, and Senftenberg, were also found in some variation between all sample sets (*71*). The serovar Rubislaw was also listed in the surface water isolates but has been commonly associated with poultry outbreaks involving turkeys and other bird species (*71*, *75*). A predominate serotype observed in the food production facility isolate group, Senftenberg, has been isolated from the reservoir poultry and has also been linked to other food commodities such as basil, beans, and animal feeds (*48*, *63*, *84*, *102*, *148*). Anatum is another serovar found in both surface water and fresh produce outbreak isolates, and associated with meat products and other food commodities such as parsley, pesto, and peanuts (*41*, *58*, *114*). Oranienburg, a serovar identified in the surface water isolated has been associated with outbreaks in fruits, cheese, cuttlefish chips, German chocolate, and black pepper (*71*, *81*, *159*). The serovars Hartford, Gaminara and Rubislaw listed in the surface water isolates have been associated with outbreaks with unpasteurized orange juice (*30*). The serovar Bareilly, identified from both Central Florida surface water and fresh produce outbreak isolates is an

amphibian-associated serotype that has also been linked to an outbreak associated with drinking water (*10*). The serovar Give was listed in the Central Florida surface water isolates and has been isolated from ruminants and pigs but is rarely found in humans (*50*, *51*). Give has also been involved in an incidence of the consumption of raw minced meat which led to a splenic abscess and an outbreak in France with infant milk formula (*51*, *76*).

The serovar Bareilly was found in both surface water and fresh produce outbreak isolates yet serotyped as the serovars Stanleyville and Infantis through Automated RiboPrinter®. Newport was also incorrectly serotyping by Automated RiboPrinter®. However, a previous study reported that both Bareilly and Newport can be serotyped via the Automated RiboPrinter using the EcoRI enzyme and were congruent with the USDA conventional methods (*7*). Some of the isolates serotyped as *S.* Newport by conventional serotyping were serotyped as *Salmonella* Newport / Bardo by Automated RiboPrinter®. *S*. Newport and *S*. Bardo have similar antigenic formulas yet differ by the presence or absence of O:6 antigen and have been assigned by the World Health Organization as a "colonial form variation" (*47*). A previous study also evaluated automated ribotyping and PFGE as rapid identification of multi-drug resistant *S*. Newport isolates and accepted the Newport/Bardo report as Newport (*45*). There were also isolates serotyped as Typhimurium by conventional serotyping and identified as *Salmonella* ser. Typhimurium /4,5,12:i:- in which S. enterica serotype I 4,[5],12:i:− is considered the monophasic variant of Typhimurium and has been indistinguishable from each other in previous studies (*7*, *121*, *167*).

The Pulse Net database proved to be a powerful tool for serotyping our isolates as mentioned in previous studies (*121*, *167*). Many of the isolates serotyped incorrectly using PFGE were examples of antigenic related *Salmonella* serovars in which the reference tool

"Antigenic Formulae of *Salmonella* Serovars" provided a great reference to observe those antigenic changes (*55*). For example, the isolates identified as serovar Flint using PFGE contained partial antigenic formula of IV Flint serovar. Serovars Nima, Florida and the R:e,n,x share partial antigenic formula from the flagellar (H) antigen with the PFGE results. However, the opposite was observed for the isolates serotyped Paratyphi B var. L-tartrate (+) using conventional serotyping where the Somatic (O) antigen formula is similar to the PFGE result. Serovar Kouka and Johannesberg share the same Phase 1 fagellar (H) antigen which might explain the PFGE result.

One serovar from the fresh produce outbreak isolated that was not identified with confidence was serotyped using conventional methods as serovar Bareilly. Barrett et al. (2006) discussed the diversity of the Bareilly serovar and a salmonellosis outbreak of *S.* Bareilly which revealed numerous patterns (*10*). An isolate from the food production facility, serotyped using conventional method as serovar Montevideo, was not identified with confidence using PFGE. In a previous study, the serovar Montevideo isolates were indistinguishable using restriction enzyme XbaI which is the same restriction enzyme used in our study (*124*). An isolate from the fresh produce outbreak serotyped using conventional method as serovar Newport but was not identified using PFGE. Results from previous studies conflict with this finding because this widely distributed serovar is commonly distinguishable by PFGE (*61*, *137*). Although the serovar Gaminara has been identified in an outbreak associated with orange juice some dissimilarities of the PFGE patterns exist between human and juice isolates (*30*).

As mentioned before, the Luminex® Salmonella serotyping assay was developed by CDC researchers, and commercialized by Luminex® company, characterizes 90% of most commonly encountered *Salmonella* serotypes in clinical settings, and is used by numerous state

public health laboratories and CDC laboratories (*43*, *95*). Previous studies have used this assay to detect diarrhea-causing bacteria including *Salmonella* enterica ser. Enteritidis and Typhimurium (*39*, *86*). Many of the isolates from Central Florida surface waters were incorrectly serotyped using xMAP assay. For example, the serovar Anatum as Wilemstad, Braenderup as Isarel, Gaminara as Schwrengrund, Harford as Irchel or Mapo/Paris, Miami as Umhlali, Stormont, Muenchen and Saintpaul as Bardo/ Newport. This shows the xMAP assay limitation in serotyping isolates from an agricultural source. One discrepancy observed in this study was the xMAP assay ability to identify the serotypes Garminara and Senftenberg in both Central Florida surface water and food production facility isolates. During the development of the assay the author recognized a limitation in that the G-complex probe for the H:t allele did not react with the t-1 probe which is the probe needed for serotype Senftenberg (*95*). Although, the serovar Gaminara was given a destination in the manual for the xMAP assay our results were inconclusive as it has been previously reported (*101*). One isolate serotyped by conventional methods as the serovar Newport, however the xMAP assay was not able to serotype this assay. A previous study also ran into inconclusive results from the Luminex serotyping assay for the serovar Newport obtained from cilantro samples from the USDA Microbiological Data Program (*12*). The serovar Oranienburg was involved in the development of the assay but our results were inconclusive for the isolate we evaluated in our study (*95*).

3.6 Conclusion

Overall, a majority of the *S.* enterica isolates with food and clinical association had a strong agreement between conventional and molecular methods. Examples of such serovars were Typhimurium, Newport, Anatum, Muenchen, and Hartford. Serotypes that are not frequently in clinical/veterinary settings proved more problematic with discrepancies between

molecular serotyping methods. The majority of those hard to define isolates were obtained from environmental sources.

CHAPTER 4
Evaluation of Contaminated Irrigation Water Affecting Transmission and Persistence of *Salmonella* spp. in Hydroponically Grown Tomatoes

4.1 Summary

Over one million foodborne illnesses cases are attributed to *Salmonella* annually in the U.S. Greenhouse/hydroponic production of tomatoes has made a significant impact on the U.S. fresh-tomato market. There is little known about the possibility of *Salmonella* contamination and internalization via greenhouse/hydroponic commercial production since these operations are usually considered relatively sanitary due to the closed environment. The objective of this study was to investigate the survival of *Salmonella* spp. in nutrient solutions used for commercial hydroponic tomato systems, as well as to determine if continuous inoculation of nutrient solution with *Salmonella* through a contaminated water source would lead to contamination in tomato fruits, leaves, roots and the formation of biofilms. An avirulent strain of *Salmonella* Typhimurium was inoculated at 10^5 CFU/mL in nutrient solution tanks of hydroponic tomato systems. Inoculation occurred on day zero and every two weeks for twelve weeks. Non-inoculated tanks served as controls. On day zero and every other day post inoculation, the nutrient solution was analyzed by plating. Leaves and biofilm samples were collected on day zero and every two-weeks post inoculation. Leaves and root samples were analyzed using enrichment and plating methods. Biofilm coupons were analyzed using a tape FISH method. Typical *Salmonella* colonies observed from leaf, fruit, and root samples were confirmed by riboprinting. There was a two-log reduction of the cells two days post-initial inoculation. Reduction of cells continued over the two-week inoculation period with few cells surviving until

the next inoculation period. Contamination was observed in the root systems. In contrast, no contamination occurred in the leaf, fruit, and biofilm samples. The results of the study show that while contaminated hydroponic nutrient solution or water leads to *Salmonella* contamination of tomato plant root, such an event may not pose a risk of *Salmonella* contamination of hydroponically grown tomato fruit.

4.2 Introduction

The 2007 Report from the Economic Research Service of the United States Department of Agriculture (USDA) reported recent increases per capita for the consumption of fresh produce (*69*). The per capita consumption of fresh produce was reported to be a little over 600 lbs. in 1980 and increased between 40 to 100 lbs. every year over a span of 30 years (*74*). This increase can be attributed to the desire for produce year round and the capacity for rapid climate controlled transportation of produce from subtropics and other hemispheres (*89*). Foodborne illness associated with fresh produce has also increased in the U.S. with an estimated 46% of foodborne illness attributed to the consumption of produce between 1998 to 2008 (*113*). Several enteric disease outbreaks have been linked to contamination of produce, such as salmonellosis from melons, tomatoes, and several varieties of sprouts; *E. coli* O157:H7 infection from leafy green vegetables; cyclosporiasis infection spread by raspberries and hepatitis A infection by green onions (*89*).

Salmonella is ubiquitous in the environment and commonly inhabits the gastrointestinal tracts of many species of domesticated and wild animals as well as humans. This genus is the leading cause of foodborne cases, resulting in 35% of hospitalizations and 28% of deaths in the U.S. attributed to foodborne illness (*127*). Although *Salmonella* originates from animal sources and has often been associated with meat and poultry outbreaks, it can also survive and colonize

in and on food crops such as leafy greens, melons, peppers, and mixed produce (*89*). According to Anderson et al. (2011) from 1986 to 2008 tomatoes ranked second in fresh produce commodities associated with foodborne pathogens and have often been linked to *Salmonella* enterica (*4*). The impact of tomato-related foodborne illness outbreaks has significantly affected the tomato industry including a $100 million dollar loss caused by a 2008 multistate outbreak of *Salmonella* Saintpaul linked to tomatoes and peppers (*99*). From 1973 to 2010 there were fifteen outbreaks of *Salmonella* linked to raw tomatoes, prompting the U. S. Food and Drug Administration (FDA) to create a "Tomato Team" to examine risk factors associated with tomatoes (*145*).

Fresh and fresh-cut produce is consumed raw with minimum processing steps to eliminate the presence of pathogens in the event of contamination (*62*). Concern has increased among consumers, growers, and retailers regarding the likelihood of contamination via environmental conditions, multiple pre- and post-harvest production factors, and exposures of susceptible populations through mishandling of produce (*89*). A potential source of spread of foodborne pathogens is handling by farm workers without access to proper latrines or hand-washing stations (*89*). Irrigation water has been highlighted as a possible source of pre-harvest contamination of tomatoes in previous studies where contact with feces from wild or domesticated animals has led to contamination (*87*, *112*). Contaminated irrigation water poses a risk not only to field-grown produce but is also a concern during greenhouse production, which may involve soilless media or hydroponic systems. Examination of the risk factors associated with greenhouse or hydroponically grown tomatoes has been examined previously (*59*, *97*). Guo et al. (2002) observed greater than 3.38 log10 CFU/g of *Salmonella* in the hypocotyls-cotyledons, stems, and leaves of plants grown for 9 days with continuous exposure to

contaminated Hoagland nutrient solution, regardless of root conditions. Hintz et al. (2010) found contamination in 65% of the roots, 40% of stems, 10% of leaves, and 6% of fruit samples after continuous contact with contaminated irrigation water. On the other hand, Miles et al. (2009) examined contamination of tomato plants after a series of alternate watering based on group number by irrigation water that contained *Salmonella* Montevideo. The researchers discovered five root samples that were positive and *Salmonella* was able to survive in the fertilizer, however, the tomato fruit was not contaminated with *Salmonella*. The risk associated with foodborne pathogens in irrigation water/nutrient solution is a major issue due to the growing tomato greenhouse industry (*87*).

There is little known about the possibility of contamination and internalization via greenhouse/hydroponic commercial tomato production since these operations are usually considered more sanitary than field operations due to the closed environment. Hence, the objective of this study was to investigate the survival of *Salmonella* in nutrient solution used for commercial hydroponic tomato systems, as well as to determine if continuous inoculation of nutrient solution with *Salmonella* through a contaminated water source would lead to contamination in fruit, leaves, or roots, and result in the formation of biofilm on the polyvinyl chloride material used throughout the system. In this study we hypothesized that continuous contamination would lead to internalization of *Salmonella* in commercially grown tomato plants, and ultimately the fruit.

4.3 Materials and Methods

4.3.1 Research Facility and Cultivar

The nutrient film technique (NFT) hydroponic system setups were located at the W.D. Holly Plant Environmental Research Center (PERC) greenhouses on the campus of Colorado State University. The cultivar grown was *Lycopersicon esculentum* (Tomato Jet Star F1, Harris seeds, Rochester, New York, USA), which was selected due to its indeterminate growth habit with a heavy vine producing large clean fruit with low resistance for cracking and scarring.

4.3.2 Tomato Growth and Hydroponic Setup

Seeds were planted into rockwool gro-blocks 6x6x5.5 inch cubes (Grodan BV, The Netherlands) and placed in trays in the PERC propagation greenhouse. At 6-weeks-old the tomato seedlings (Jet-Star) were trimmed and transplanted into and allowed to root throughout net pots (American Clayworks, Denver, CO, USA). The seedlings were staked for support using garden stakes (Bond Manufacturing, Antioch, California, USA).

The net pots were distributed throughout a NFT hydroponic system (8 tomato plants per system). NFT channels were selected due to their ability to be a closed environment to address biosafety concerns. There were six independent commercial NFT hydroponic systems constructed. The NFT systems were divided into two treatments groups: control (nutrient solution contained deionized water only) and experimental (nutrient solution contained deionized water contaminated with *S.* Typhimurium every 2 weeks). Each system was composed of a 300-gallon nutrient reservoir (donated by New Belgium Brewery, Fort Collins, Colorado, USA), 25-gallon re-circulating return reservoir (Rubbermaid, Atlanta, Georgia, USA), and 2- GT50 TSW

series NFT hydroponic channels and accessories (4 x 2 inches, Grow Tech, Dyersville, Iowa, USA) contained in Ebb and Flow table and covered with plastic. The nutrient tank was filled to 150-gallons using a volumetric shut off valve (Bermadon brand, FarmTek, Dyerville, Iowa, USA). Each nutrient tank contained a thermometer, air stone, EC, and pH probe monitor which were sealed in place for daily tank measurements. The reservoirs and channels were constructed and sealed with 1½ inch and 2 inch polyvinyl chloride (PVC, JM Engle, Los Angeles, California, USA), PVC Primer and cement (Weld-on brand, IPS Corporation, Compton, California, USA), silicone (GC Brand for Kitchen & Bath, Momentive, Columbus, Ohio, USA), plumbing putty epoxy (Protective Coating [PC] Company, Allentown, Pennsylvania, USA) and LeakSeal Clear spray (Rust-Oleum Corporation, Vernon Hills, Illinois, USA). The nutrient reservoirs were painted with black spray paint (Rust-Oleum Corporation, Vernon Hills, Illinois, USA) to reduce the production of algae. The NFT system was a recirculating system in which a waterfall pump (Flotec, Delavan, Wisconsin, USA) located in the nutrient reservoir was used to pump nutrients through the hydroponic channel where the run-off was collected in the return tank and nutrients pumped back to the nutrient reservoir using a water removal utility pump with on/off intelligence (Flotec, Delavan, Wisconsin, USA). Once the plants were transplanted into the NFT system, they were staked using a string and zip ties to a bar above each system. The systems were slanted at a 2% grade for gravity flow.

4.3.3 Maintenance of Nutrients and System Care

The nutrients used in the nutrient solution were Chem-Gro Tomato Formula 4-18-38 (Hydro-Garden Inc., Colorado Springs, Colorado, USA), calcium nitrate (Norsk Hydro Agri North America, Inc., Tampa, Florida, USA), magnesium sulfate (Hydro-Garden Inc., Colorado

Springs, Colorado, USA), pH up and pH down concentration (Advanced nutrients, Abbotsford, B.C, Canada, USA). The nutrients levels were maintained as an EC level of 1.0 and a pH level of 6.0 and monitored and recorded daily. Oxygen was incorporated in the system using an air pump with air stones/diffuser (ActiveAqua-Hydrofarm, Petaluma, California, USA). The pH and electric conductivity (EC) were measured consistently using monitors (Milwaukee Instruments, Rocky Mount, North Carolina, USA). Both monitors (pH and EC) were calibrated weekly and calibrated according to manufacturer instructions. The temperature was monitored using an aquarium digital thermometer (Petco, San Diego, California, USA) and recorded daily. Pollination was done by hand with daily maintenance of the plants by shaking the flower cluster. No pesticides were used during this experiment. The temperature and relative humidity of the greenhouse facility was maintained using the Wadswoth Control System (Arvada, Colorado, USA).

4.3.4 Biosafety

All biosafety concerns were addressed with the Colorado State University Environmental Health Services Biosafety Office. The NFT system was used for this study to address biosafety concerns about aerosolization of the *Salmonella* spp. used in this study. Each NFT system was sealed and checked for leaks daily. Environmental samples were taken every two weeks using a ready-to-use environmental swab system (3M™ Quick Swabs, Saint Paul, MN, USA). The greenhouse facility was divided with a sealed plastic wall into two areas: preparation area and research area. The preparation area housed the hand and foot wash stations, personal protection equipment (PPE) storage, nutrients and materials used to maintain the NFT system. The research area housed the NFT systems. Before entering and exiting the research area the researchers were

required to step in a footbath containing 70% bleach solution (made fresh daily). Researchers were required to wear the following PPE: Tyvek coverall (DuPont Tyvek Isoclean, VWR, Randor, Pennsylvania, USA), N95 respirator (VWR, Randor, Pennsylvania, USA), face shield (Nalgene ® VWR, Randor, Pennsylvania, USA), rubber nitrile gloves (Midknight®, Reno, Nevada, USA), and rubber boots (Servus ™,VWR, Randor, Pennsylvania, USA).

4.3.5 Bacterial culture and antimicrobial susceptibility

The avirulent strain of *Salmonella* Typhinurium (*Salmonella* LT2) was provided by Dr. Michelle Danyluk, University of Florida, for use in this study. The strain was stored at -80°C in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 20% glycerol. *S.* Typhimurium LT2 strain was tested with a panel of 12 antimicrobials using the Kirby-Bauer disk diffusion method at Colorado State University Veterinary Diagnostic Laboratory (Fort Collins, CO). The strain was found to be resistant to amikacin, ceftiofur, cephalothin, gentamicin, and streptomycin. The median inhibitory concentration (MIC) score was used to determine the proper concentration for growth of the pathogen on selective media and pre-enrichment broth. Selective medias and broths used throughout the research were supplemented with gentamicin (Sigma-Aldrich, Saint Louis, MO, USA) and streptomycin (Sigma-Aldrich, Saint Louis, MO, USA) at ½ MIC concentration for XLD agar plates based on the MIC concentration.

4.3.6 Inoculum preparation and spiking

To begin each spiking experiment, a frozen strain (*S.* Typhimurium LT2) was activated by streaking on tryptic soy agar (TSA, Becton Dickinson, Sparks, MD, USA) and incubated at 35±2

°C for 24 hours. One isolated colony was selected and transferred to 10 mL of tryptic soy broth (TSB, Becton Dickson, Spark, MD, USA) followed by incubation at 35±2 °C for 24 hours. Ten milliliters of overnight culture were transferred to 300 mL of TSB and incubated at 35±2 °C for 24 hours. A 10-milliters aliquot of the overnight inoculum was removed for enumeration on TSA, to confirm inoculum concentration using dilution plating of desired concentration of 9 log CFU•mL-1. Three hundred milliliters of the overnight culture were re-suspended into 150-gallons nutrient solution to create a final inoculum of 5log CFU•mL-1. Inoculation of the nutrient solution occurred on day zero and every 2-weeks for 12-weeks. Non- inoculated tanks served as controls.

4.3.7 Enumeration of nutrient solution

One hundred and twenty milliliters of nutrient solution were pumped into a sterile specimen cup (4 oz., VWR Microbiology/Urinalysis Specimen containers, VWR, Randor, PA). The samples were stored on ice and transported to the laboratory. Twenty milliliters of the nutrient solution were removed for serial dilution. One hundred milliliters of nutrient solution was serially diluted in lambda buffer (100mL NaCl, 8mM $MgSO_4 \cdot 7H_2O$, 50mL Tris-HCl [pH7.5]) and filtered through a beverage filter (Microcheck beverage filter, Pall® Life Sciences, Port Washington, NY) according to manufacturer's instructions. The filter was placed face up on a XLD agar plate (HiMedia, VWR, Randor, PA) with supplements and incubated at 35±2 °C for 24 hours. Colonies were enumerated to determine concentration in CFU•mL-1. Samples collection occurred on day zero and every other day post initial inoculation.

4.3.8 Tomato plant sampling

Portions of the tomato plants were randomly sampled for the presence of *S.* Typhimurium. Leaf samples were collected on day zero and every two weeks. Tomato fruit samples were collected six weeks post initial inoculation of hydroponic system. Root samples were collected at the termination of the twelve-week project post initial inoculation.

4.3.9 Leaf analysis

Four mature leaves were randomly selected from the upper and lower portion of the tomato plant. The leaves from each portion were pooled to create one individual sample. The 4 leaves were aseptically removed from the plant using sterilized scissors and transferred into a single sterile stomacher bag (Nasco Whirl-Pak, Fort Atkinson, WI). Samples were weighed and a 1:5 test portion of broth ratio (weight to volume) of buffered peptone water (BPW, Becton Dickson, Spark, Maryland) with supplements was added to stomacher and stomached for 90s. Samples were incubated at 35±2 °C for 24 hours. The overnight enrichment was streaked onto XLD agar (HiMedia, VWR, Randor, Pennsylvania) with supplements. Following 24 hour incubation at 35±2 °C, typical *Salmonella* colony formation was considered presumptive positive.

4.3.10 Tomato fruit analysis

All green to orange tomato fruits were harvested from the tomato plants. Tomato fruits were aseptically removed from the plant using sterilized scissors and transferred into individual sterile stomacher bags. The total amount of samples collected was split in half. One half of the tomatoes were analyzed using the modified whole soak method and the other half were analyzed

using the modified quarter/stomach method. For the whole soak method, the tomato fruit were weighed and a 1:1 test portion of broth ratio (weight to volume) of BPW (Becton Dickson, Spark, Maryland) with supplements was added to the stomacher bag. For the quarter/stomach method, the tomato fruit was weighed, aseptically quartered along the stem line, transferred aseptically into individual stomacher bags, and a 1:1 test portion of broth ratio (weight to volume) of BPW (Becton Dickson, Spark, Maryland) with supplements was added to the stomacher bag. The samples were soaked at 4°C for 24 hours and transferred to be 35±2 °C incubator for 24 hours. The overnight enrichment was streaked onto XLD agar (HiMedia, VWR, Randor, Pennsylvania) with supplements. Following 24 hour incubation at 35±2 °C, typical *Salmonella* colony formation was considered presumptive positive.

4.3.11 Root analysis

The bottom portion of the tomato plant, including net pots and the root system, were aseptically removed from the NFT system using scissors. Each root system was individually placed in a stomacher bag and weighed. A 1:1 test portion of broth ratio (weight to volume) of BPW (Becton Dickson, Spark, MD, USA) with supplements was added to the stomacher bag. Samples were incubated at 35±2 °C for 24 hours. The overnight enrichment was streaked onto XLD agar (HiMedia, VWR, Randor, PA) with supplements. Following 24 hour incubation at 35±2 °C, typical *Salmonella* colony formation was considered presumptive positive.

4.3.12 Automated RiboPrinter

The typical *Salmonella* colonies observed from the leaves, fruit, and root samples were confirmed using automated RiboPrinting. Ribosomal DNA from each isolate was profiled using

the RiboPrinter Microbial Characterization System (Dupont Qualicon, Wilmington, Delaware) in accordance with the manufacturer's instructions. Briefly, single colonies from overnight tryptic soy agar cultures were selected, suspended in sample buffer, and heat-treated before being placed in the automated RiboPrinter Microbial Characterization System. Restriction was then performed with PvuII. In this system, the restricted DNA is separated by gel electrophoresis and subsequently transferred to a nylon membrane for Southern blot analyses using DNA probes complementary to ribosomal sequences, yielding a Riboprint pattern. The Riboprint patterns of the isolates were matched to reference patterns using DuPont RiboExplorer Software (Ver. 2.2.0232.0).

4.3.13 Biofilm analysis

PVC type I gray coupons (2.5 cm x 2.5 cm, Fort Collins Plastics, Fort Collins, Co) were hung from the lid of the re-circulating return reservoir with string and weighed down using a stainless steel nut (Handiman, Walmart, Bentonville, AR). The surface of the coupon were analyzed for the presence of *Salmonella* spp. using the Tape Fluorescence in situ Hybridization (FISH) methodology as described by Bisha and Brehm-Stecher (*21*). Microscopic analyses was performed on Leica DM4500 P LED microscope (Leica Camera, Germany). Images analyses were captured using the Q-Capture Pro7 (Q Imaging, Canada).

4.3.14 Statistical Analysis

Each treatment group (*S.* Typhimurium-irrigated and control) contained 8 tomato plants (total of 48 plants). Leaves and fruit from each group were sampled at random. The correlation between the plant tissue type (root, leaves, or fruit) and control (uninoculated) on the presence of

S. Typhimurium was evaluated using χ^2 analysis. Statistical analysis was completed using R Foundation for Statistical Computing (R version 2.15.1).

4.4 Results

4.4.1 Survival of avirulent *Salmonella* Typhimurium in nutrient solution

There were 4 treated systems (nutrient solution inoculated at log 5 CFU/mL) and 2 control systems (uninoculated). Over the course of the experiment, the desired level of *S.* Typhimurium at log 5 CFU/mL was obtained in the nutrient solution at every spiking event (Figure 4.1). No *S.* Typhimurium was recovered from the control tanks. There was an initial 2-log reduction observed between the initial spiking event and 2 days prior. The reduction continued until the next spiking event. This suggested that *S.* Typhimurium did not survive well in the conventional nutrient solution.

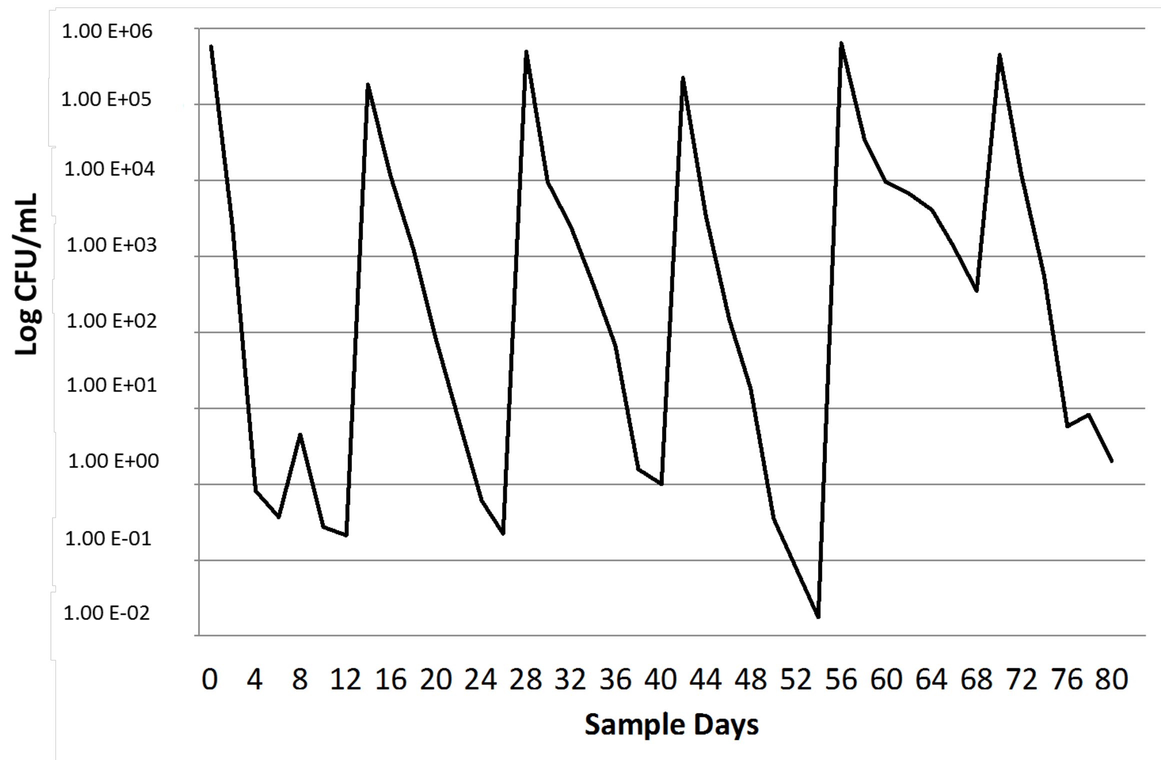

Figure 4. 1 Survival of avirulent *Salmonella enterica* serovar Typhimurium LT2 in nutrient solution. Control readings not shown due to growth not observed.

4.4.2 Tomato plant analysis

There was a total of 32 tomato plants in the treated hydroponic systems and 16 tomato plants in the control (uninoculated) hydroponic systems. Tomatoes samples were collected 6 weeks post initial inoculation and every week until the termination of the project at 12 weeks. No presumptive *S.* Typhimurium colonies were observed from any of the tomato samples taken from both treated and control plants (Table 4.1). At the start of week 8 one of the treated hydroponic system (tank C) plants collapsed and completely damaged the hydroponic setup. The system was repaired but the plants were damaged and difficult to reposition. One of the treated tomato samples from the lower portion of a plant tested positive to *S.* Typhimurium (Table 4.1). Leaf samples were obtained from upper and lower portions of the plant and pooled for analysis on day

0 and every 2 weeks. At week 11 a sample from the lower portion of a tomato plants in a treated hydroponic system (tank C) tested positive for *S.* Typhimurium (Table 4.1). The accident, which occurred at week 8, was unforeseen and both positive samples were suspected to have contact with the contaminated nutrient solution. No presumptive *S.* Typhimurium colonies were observed from any of the leaf samples taken from both treated and control plants. However, presumptive *S.* Typhimurium was observed from 10 of the 23 (43%) of the root samples. There was a significant difference between control and treatment of roots ($P < 0.05$). All presumptive samples were confirmed using Automated RiboPrinting.

Table 4. 1 Number of tomato plant samples [tomato fruit (quartered and whole), leaves (upper and lower), and roots] positive and negative results from presumptively *Salmonella enterica* serovar Typhimurium samples confirmed using Automated RiboPrinting ®.

	Quatered Tomatoes		Whole Tomatoes		Upper Leaves		Lower Leaves		Roots[a]	
	C	T	C	T	C	T	C	T	C	T
Positive	0	1	0	0	0	0	0	1	0	10
Negative	85	79	97	101	112	224	112	223	16	23
Total	85	80	97	101	112	224	112	224	16	32

C= Control Samples, T= Treated Samples

[a] Significant difference observed by χ^2 analysis ($P < 0.05$)

DuPont ID Similarity	DuPont ID Label	RiboPrint™ Pattern 1 kbp 5 10 15 50
0.95	Salmonella ser. Typhimurium	
0.96	Salmonella ser. Typhimurium	
0.89	Salmonella ser. Typhimurium	
0.89	Salmonella ser. Typhimurium	
0.93	Salmonella ser. Typhimurium	
0.92	Salmonella ser. Typhimurium	

Figure 4. 2 Automated RiboPrinter® patterns using the restriction enzyme *Pvu*II from the stock isolate and five of the root samples.

4.4.3 Biofilm analysis

Tape FISH analyses on PVC coupons were performed as described by Bisha and Brehm-Stecher 2010 (*21*). The probes Sal3 and Sal63 (Integrated DNA Technologies, Coralville, IA) were used to label the *S.* Typhimurium cells. No presumptive *S.* Typhimurium colonies were observed on the PVC coupons from both treated and control tanks (Figure 1 & 2). Positive controls were also analyzed. The coupons were also enriched in BPW and streaked out onto XLD agar and typical colonies were not observed.

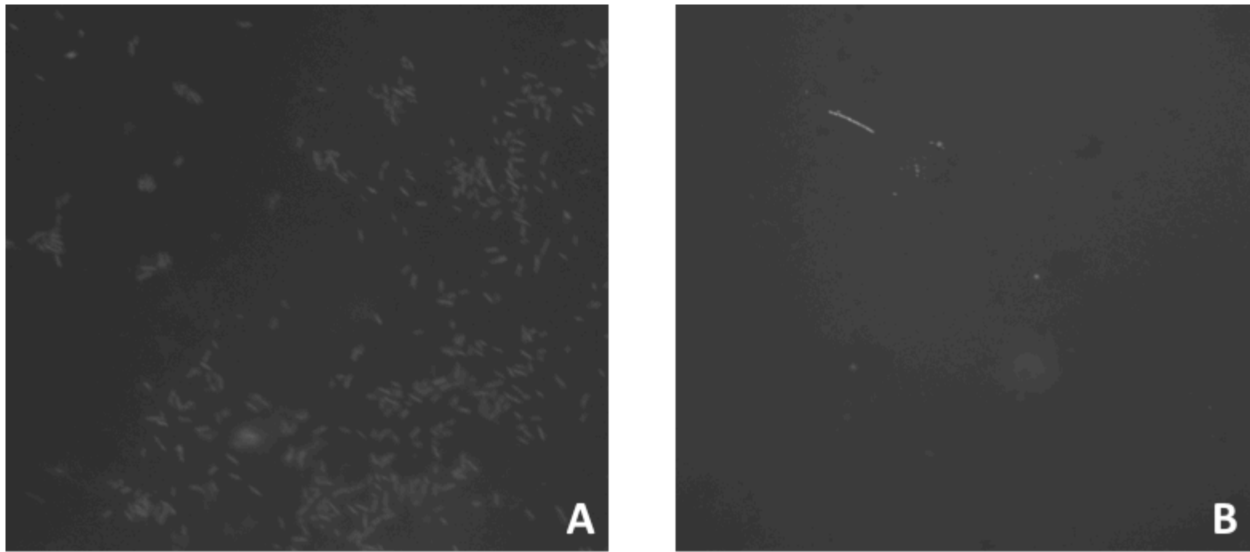

Figure 4. 3 A and B. Figure A. Typical results for direct-to-tape sampling and FISH detection of *Salmonella enterica* serovar Typhimurium on positive control (40X objective, two-probe cocktail of Cy3-labeled probes-Sal3/Salm-63). Figure B: Typical results for direct-to-tape sampling and FISH detection of *Salmonella enterica* serovar Typhimurium on treated tank B (negative control).

4.5 Discussion

Salmonella enterica has been consistently linked to tomato-associated outbreaks. According to Jackson et al. (2013) the serovars associated with tomato outbreaks are Newport (29%), Typhimurium (16%), Braenderup (11%), Enteritis (11%) and Javiana (11%) (*71*). Among those serovars, Typhimurium and Newport are widely distributed throughout the environment. Specifically, Typhimurium has the ability to infect various species including food animals such as poultry and pigs and surviving for long periods of time (*71*, *120*). The survival of *S*. Typhimiurim in tomatoes has been examined in previous studies (*25*, *56*). Gu et al. (2011) observed that *S*. Typhimurium could be internalized into tomato plants via leaves inoculated with suspension of 10^9 CFU/ml *Salmonella* with surfactant Silwet L- 77 without inducing any symptoms in the tomato plants (*56*). In our study we did observe contamination on a single tomato fruit and leaf samples after the accident with the system which makes us ask if the presence of Typhimurium could lead to post harvest contamination. Cevallos-Cevallos et al.

(2012) found that Typhimurium may be dispersed by rain to contaminate tomato plants in the field particularly at 10 min events and of plastic mulch is used (*25*). In our study we did observe contamination on a single tomato fruit and leaf samples after the accident with the system which makes us ask if the presence of Typhimurium could lead to post harvest contamination (*165*). In one study, the researchers used serovars associated in tomato-linked outbreaks of salmonellosis (Javiana, Montevideo, and Newport) and those typically isolated from animal or clinical infection (Dublin, Enteritidis, Hadar, Infantis, Typhimurium and Seftenberg) to test the survival from the inoculated flowers of growing plants (*134*). It was discovered that Montevideo was more adaptive to the tomatoes and was recovered 90% of the time and all serovars were able to grow and become established in unripened green tomatoes. On the other hand, the growth in ripened tomatoes was serovar dependent in which serovars Enteritidis, Typhimurium and Dublin were less adaptive to grow on ripen intact tomatoes.

In this study we observed *S.* Typhimurium might lead to contamination of the root system but not the leaves or tomato fruits when introduced through contaminated nutrient solution. Hintz et al. (2010) also observed that *S.* Newport may be associated with the root system and to a lesser degree with the stem and leaves of tomato plants when introduced to contaminated irrigation water (*65*). Nevertheless, there are some instances when inoculation of the root may lead to contamination especially in the presence of a plant pathogen such as *R. solanacearum* (*119*). Guo et al. (2002) has shown a hydroponic system with direct root inoculation with *S.* enterica could lead to contamination of the entire plant (*59*). Guo et al. (2001) also discovered that *Salmonella* could survive in or on tomato fruit from the time of inoculation at the flowering stage through the fruit (*60*). Miles et al. (2009) observed survival of *S.* Montevideo in five of their root samples and all tomato fruits tested negative (*97*). On the other hand, Montevideo was

found to be able to survive in the commercially available fertilizer used in the study. Barak et al. (2011) found plants irrigated with contaminated water had larger populations of *Salmonella* than those grown in infested soil (*8*). Although Barak et al. (2008) did observe that *Salmonella* enterica can survive up to six weeks in fallow soil with the ability to contaminate the tomato plant and that soil contamination could lead to contamination of the tomato phyllosphere (*9*).

One of our major objectives in this study was to determine the survival of *Salmonella* spp. in our conventional nutrient solution. We observed that the survival of *S.* Typhimurium in the nutrient solution of our hydroponic systems was low. However, in the United States the major serovars associated with waterborne salmonellosis includes Typhimurium, Enteritidis, Bareilly, Javiana, Newport and Weltevreden (*29*, *68*). Typhimurium has been consistently linked to gastroenteritis outbreaks in the United States (*29*). Typhimurium was also linked to a major outbreak in drinking water in Alamosa, Colorado in 2008, costing local, state, and nongovernmental agencies and the city of Alamosa healthcare facilities and schools millions of dollars (*2*).

In our study we also did not observe any formation of biofilm on the PVC coupons collected from the recirculating tank. However, Zacheus et al. (1999) found that bacterial biofilm formation was higher on polyvinyl chloride (PVC) than on (PE) (*162*). Previous studies have shown that the flagella plays a major role in biofilm formation and the attachment to PVC surfaces (*77*, *98*, *138*). Biofilms are defined as communities of bacterial cells enclosed in a self-produced polymeric matrix to inert or survive in most biological systems (*78*, *139*). Its formation and the accumulation of microorganisms on surfaces depends on various factors such as surface materials, microbial occurrence in water, concentration, temperature, hydraulics of systems, concentration/quality of nutrients and disinfectants (*162*). In our study we observed

that the *Salmonella* did not survive well in the nutrient solution so that could have been a factor in the absence of biofilm formation. Typically, biofilms are common in aquatic environments such as recirculating systems because they and harbor pathogenic organisms through the water/solid interface on tanks and equipment (*78*, *162*).

4.5 Conclusion

Overall, we observed that after continuous contamination of *S.* Typhimurium infected nutrient solution might be capable of contaminating the root site of the tomato plants. We were not able to recover *S.* Typhimurium from the other portions of the plants tested, however we did observe post-harvest contamination after a system was damaged. *S.* Typhimurium also did not survive well in the conventional nutrient solution used in the study and biofilm formation was not observed.

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APPENDIX

Appendix 1. 1 Antimicrobial susceptibility for avirulent strain of *Salmonella enterica* serovar Typhimurium (STL-2 MDD14) report from the Colorado State Veterinary Diagnostic Laboratory (Page 1of 2).

300 West Drake Road
Fort Collins, CO 80523-1644
(970) 297-1281

Laboratory Report
Final

This report supersedes all previous reports for this case

Case #: F1338412
Referral #: ST L 2 MDD14
Date Collected:
Date Received: 05/29/2013
Case Coordinator: Dr. Doreene Hyatt
Owner:
Coleman
FORT COLLINS

Email To: linda.moller@colostate.edu
5314200
1171 Animal Sciences
Attn: Linda Moller
FORT COLLINS, CO 80523

Electronically Signed and Authorized By:
Dr. Doreene Hyatt
sent by Denise Bolte
on 5/30/2013 12:44:04PM

Case Contacts

Report To	Coleman, Shannon	205-527-2946	smcolema@rams.colostate.edu
Submitter	5314200	9704911441	linda.moller@colostate.edu

Specimen Details

ID	Taxonomy	Sex	Age
STL2 MDD14			

Owner: Coleman
Specimens Received: Bacterial Isolate;

Bacteriology

Antibiotic Susceptibility

Animal/Source	Specimen	Specimen Type	Result Date	Results
STL2 MDD14	1	Bacterial Isolate	30-May-2013	Complete Salmonella Group B

Standard Report(m) - 7/22/2010

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Appendix 1. 2 Antimicrobial susceptibility for avirulent strain of *Salmonella enterica* serovar Typhimurium (STL-2 MDD14) report from the Colorado State Veterinary Diagnostic Laboratory (Page 2 of 2).

CSUVDL Final
Owner: Coleman

Accession # F1338412

May 30, 2013

Appendix - Report Related Images

Colorado State University Veterinary Diagnostic Laboratory Thursday, May 30, 2013
300 West Drake Road
Fort Collins, CO 80523
970-297-0327
It is the responsibility of the laboratory client to ensure that compounds are used appropriately for certain species or age groups of animals.

Specimen Date	Tuesday, May 28, 2013		
Specimen Number	F1338412	Isolate Number	1
Patient	COLLMAN,		
Location	Other	Specimen Type	MISCELLANEOUS
Organism	Salmonella species	Doctor	MOLLEE
Comment	GROUP B		

ANTIBIOTICS	CATEGORY	MIC (mcg/ml)
Amikacin	R	4
Amoxicillin-clavulanate	S	6
Ampicillin	S	2
Ceftiofur	R	2
Cephalothin	R	4
Chloramphenicol	S	6
Enrofloxacin	S	0.125
Gentamicin	R	1
Streptomycin	R	12
Sulfonamides	S	<48
Tetracycline	S	6
Trimethoprim-sulfamethoxazole	S	6

The client assumes all responsiblity for efficacy, safety, and residue avoidance.
F1338412 (1) COLEMAN, MISCELLANEOUS Other

End of Report

Standard Report(m) - 7/22/2010

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Appendix 1. 3 All comparative serotyping data for Central Florida surface water isolate

Central Florida surface water isolates				
Isolate identifier	**Conventional serotyping result**	**PFGE result**	**xMAP assay result**	**Riboprinting ID**
70	III_17:z10:e,n,x,z15	Could not be identified with confidence	Could not be identified with confidence	Could not be identified with confidence
111	III_44:z4,z32:-	Could not be identified with confidence	Could not be identified with confidence	Salmonella IIIa
113	III_44:z4,z32:-	Could not be identified with confidence	Could not be identified with confidence	Salmonella IIIa
367	IV_50:z4,z23:-	Flint	Tejas/Vuadens/Wilhelmsburg/Stanleyville	Salmonella IV
150	Rough_O:d:1,7	Florida	Could not be identified with confidence	Salmonella ser. Kottbus
151	Rough_O:d:1,7	Florida	Could not be identified with confidence	Salmonella ser. Farmsen
274	Rough O: y:1,5	Nima	Could not be identified with confidence	Salmonella ser. Miami
281	Rough O: y:1,5	Nima	Could not be identified with confidence	Salmonella ser. Perth
62	IV Rough O:z4,z24:-	Salmonella subspecies IV	Could not be identified with confidence	Could not be identified with confidence
63	IV Rough O:z4,z24:-	Salmonella subspecies IV	Could not be identified with confidence	Could not be identified with confidence
74	IV Rough O:z4,z23:-	Could not be identified with confidence	Could not be identified with confidence	Could not be identified with confidence
119	IV_50:z4,z23:-	Flint	Could not be identified with confidence	Could not be identified with confidence
312	6,8:d:-	Could not be identified with confidence	Could not be identified with confidence	Could not be identified with confidence
543	6,8:d:-	Could not be identified with confidence	Could not be identified with confidence	Salmonella III
202	50:z4,z23:-	Flint	Could not be identified with confidence	Could not be identified with confidence
92	Anatum	Anatum	Willemstad	Salmonella ser. Anatum
130	Anatum	Anatum	Willemstad	Salmonella ser. Anatum
217	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
331	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
332	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
334	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
304	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
674	Anatum	Anatum	Willemstad	Salmonella ser. Anatum
711	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
745	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
341	Anatum	Anatum 100%	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
190	Baildon	Baildon	Baildon/Lomalina/ ll[1],9,12:a:e,n,x	Salnonella ser. Stanley
192	Baildon	Baildon	Baildon/Lomalina/ ll[1],9,12:a:e,n,x	Salmonella ser. Baildon
194	Baildon	Baildon	Baildon/Lomalina/ ll[1],9,12:a:e,n,x	Salmonella ser. Stanley
195	Baildon	Baildon	Baildon/Lomalina/ ll[1],9,12:a:e,n,x	Salmonella ser. Baildon
216	Bareilly	Bareilly	Could not be identified with confidence	Salmonella ser. Stanleyville
582	Braenderup	Braenderup	Braenderup	Salmonella PVUII Group #1
592	Braenderup	Braenderup	Braenderup	Salmonella ser. Bareilly
597	Braenderup	Braenderup	Could not be identified with confidence	Salmonella ser. Enteritidis
604	Braenderup	Braenderup	Israel	Salmonella ser. London/Braenderup
609	Braenderup	Braenderup	Israel	Salmonella ser. Paratyohi B.
156	Braenderup	Braenderup 100%	Israel	Salmonella ser. Bareilly
124	Florida	Could not be identified with confidence	Could not be identified with confidence	Salmonella ser. Kottbuss
210/220	Florida	Could not be identified with confidence	Could not be identified with confidence	Could not be identified with confidence
306	Gaminara	Could not be identified with confidence	Could not be identified with confidence	Salmonella ser. Lille
22	Gaminara	Hadar	Could not be identified with confidence	Salmonella ser. Gaminara
26	Gaminara	Hadar	Could not be identified with confidence	Salmonella ser. Lille
49	Gaminara	Gaminara	Could not be identified with confidence	Salmonella ser. Lille
97	Gaminara	Gaminara	Could not be identified with confidence	Salmonella ser Lille
98	Gaminara	Gaminara	Could not be identified with confidence	Salmonella ser Gaminara
117	Gaminara	Gaminara	Could not be identified with confidence	Salmonella ser. Lille
140	Gaminara	Bareilly	Could not be identified with confidence	Salmonella ser. Lille
144	Gaminara	Gaminara	Could not be identified with confidence	Salmonella ser. Lille
158	Gaminara	Gaminara	Could not be identified with confidence	Salomella ser. Lille
616	Gaminara	Bovismorbificans	Could not be identified with confidence	Salmonella ser. Enteritidis
620	Gaminara	Bovismorbificans	Could not be identified with confidence	Salmonella ser. Enteritidis
614	Gaminara	Bovismorbificans	Schawrzengrund	Salmonella ser. Lille
168	Gaminara	Gaminara	Could not be identified with confidence	Salmonella ser. Lille
77	Georgia	Could not be identified with confidence	Mana	Could not be identified with confidence
677	Give	Could not be identified with confidence	Could not be identified with confidence	Could not be identified with confidence
132	Hartford	Hartford	Irchel	Salmonella ser. Hardford
690	Hartford	Hartford 92%	Hartford	Salmonella ser. Hartford
691	Hartford	Hartford 92%	Hartford	Salmonella ser. Hartford
68	Inverness	Inverness	Could not be identified with confidence	Salmonella ser. Aqua/Inverness
157	Ituri	Ituri 96%	Mapo/Paris	Could not be identified with confidence
714	Ituri	Ituri	Ituri	Could not be identified with confidence
326	Litchfield	Litchfield	Could not be identified with confidence	Could not be identified with confidence
66	Miami	Miami	ll 9,12:a:1,5/Sendai/Miami	Salmonella ser. Miami
131	Miami	Miami	Umhlali	Salmonella ser. Miami
134	Miami	Miami	Umhlali	Salmonella ser. Miami
305	Muenchen	Muenchen	Virgina/Muenchen	Salmonella ser. Muenchen
343	Muenchen	Muenchen 90%	Virgina/Muenchen	Salmonella ser. Muenchen
136	Muenchen	Muenchen	Stormont	Salmonella ser. Muenchen
147	Muenchen	Muenchen	Virgina/Muenchen	Salmonella ser. Muenchen
165	Muenchen	Muenchen	Stormont	Salmonella ser. Anatum
430	Muenchen	Cotham	Virgina/Muenchen	Salmonella ser. Muenchen
751	Muenchen	Muenchen	Virgina/Muenchen	Salmonella ser. Muenchen
754	Muenchen	Muenchen 90%	Bardo/Newport	Salmonella ser. Muenchen
15	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
101	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
435	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
537	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
659	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
145	Norwich	Norwich	Could not be identified with confidence	Could not be identified with confidence
753	Norwich	Norwich	Could not be identified with confidence	Could not be identified with confidence
161	Oranienburg	Oranienburg 100%	Could not be identified with confidence	Salmonella ser. Oranienburg
29	Paratyphi_B_var.L-tartrate+	Paratyphi B	Could not be identified with confidence	Salmonella ser. Paratyphi B Var. L (+)
369	Paratyphi_B_var._L-tartrate+	I 4,[5],12:b:- 96%	Could not be identified with confidence	Salmonella ser. Paratyphi B Var. L (+)
163	Rubislaw	Rubislaw	Rubislaw/Kibusi	Salmonella ser. Elomrane
164	Rubislaw	Rubislaw	Rubislaw/Kibusi	Salmonella ser. Elomrane
250	Rubislaw	Rubislaw 92%	Rubislaw/Kibusi	Salmonella ser. Elomrane
615	Rubislaw	Rubislaw 93%	Rubislaw/Kibusi	Salmonella ser. Elomrane
749	Rubislaw	Rubislaw	Rubislaw/Kibusi	Salmonella ser. Elomrane
752	Rubislaw	Rubislaw	Rubislaw/Kibusi	Salmonella ser Elomrane
83	Rubislaw	Rubislaw	Rubislaw/Kibusi	Salmonella ser. Elomrane
129	Saintpaul	Saintpaul 100%	Bardo/Newport	Could not be identified with confidence
744	Saintpaul	Saintpaul	Saintpaul	Salmonella ser. Saintpaul
231	Typhimurium var 5-	Typhimurium	Typhimurium var. 5/Typhimurium	Salmonella ser. Typhimurium

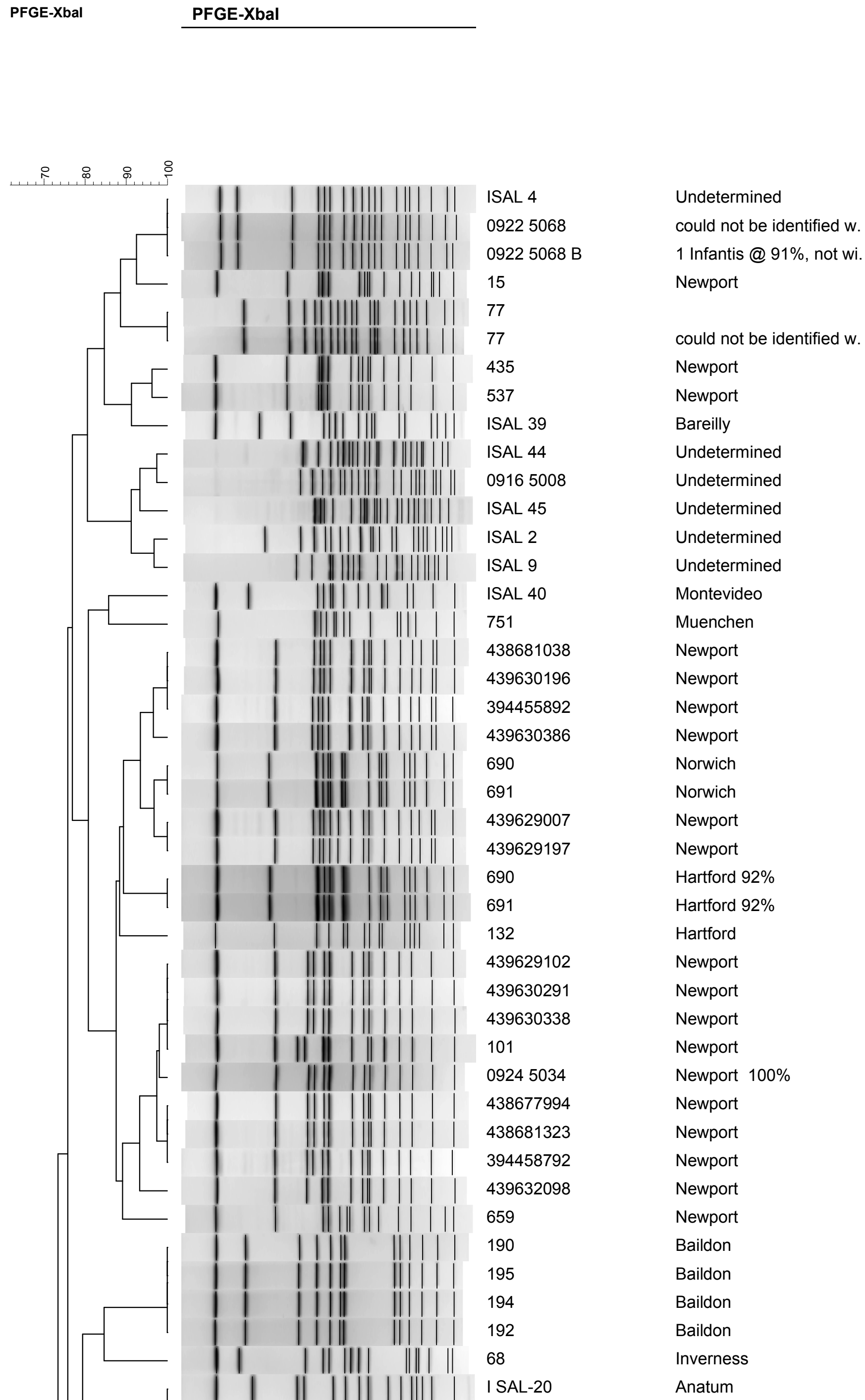
Appendix 1. 4 All comparative serotyping data for food production facility isolates.

Food production facility isolates				
Isolate identifier	Conventional serotyping result	PFGE result	Bioplex result	Riboprinting ID
1006 5010	L: e,n,x	Senftenberg	Could not be identified with confidence	Could not be identified with confidence
0916 5005	Kouka	Johannesburg	Senftenberg	Salmonella ser. Senftenberg
0916 5005	Montevideo	Could not be identified with confidence	Montevideo	Could not be identified with confidence
0922 5154	R:e.n.x	Johannesburg	Johannesburg / Urbana	Salmonella ser. Johannesburg/Urbana
1010 5007	R:e,n,x	Johannesburg	Johannesburg / Urbana	Salmonella ser. Johannesburg/Urbana
0916 5008	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg
1007 5008	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg
0916 5009	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg
0915 5011	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg
1011 5011	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg
1012 5012	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg
0914 5013	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg
0915 5014	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg
0916 5014	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg
1009 5014	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg
0915 5015	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg
0916 5016	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg
1009 5018	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg
0922 5151	Senftenberg	Senftenberg	Could not be identified with confidence	Salmonella ser. Senftenberg

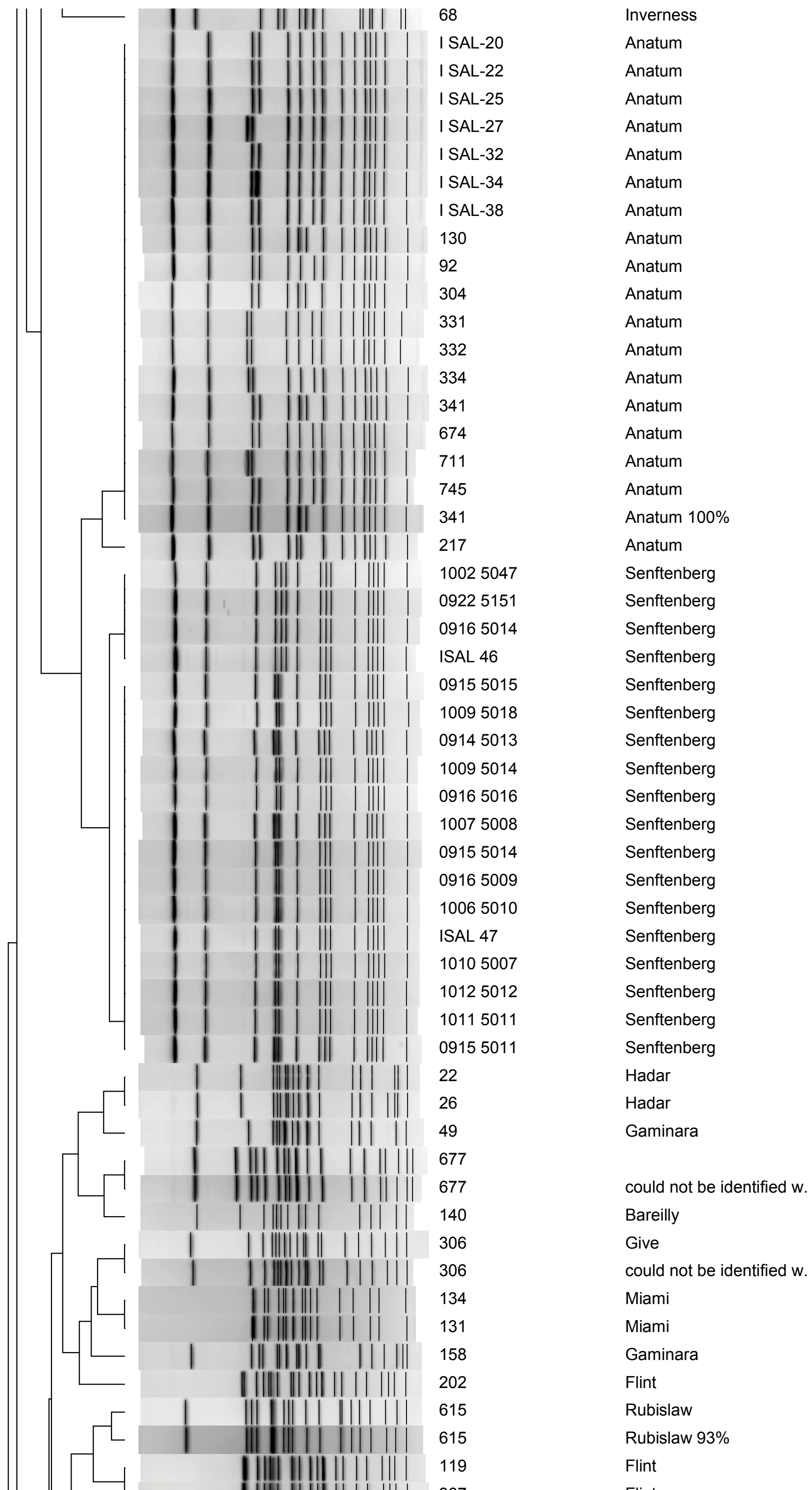
Appendix 1. 5 All comparative serotyping data for fresh produce outbreak isolates.

Fresh produce outbreak isolates				
Isolate identifier	Conventional serotyping result	PFGE result	Bioplex result	Riboprinting ID
0924 5030	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
0924 5033	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
0924 5039	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
0924 5056	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
0924 5062	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
0924 5095	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
0924 5140	Anatum	Anatum	Hayindogo/Anatum var. 15+,34+/Anatum var. 15+/ Anatum	Salmonella ser. Anatum
0922 5068	Bareilly	Could not be identified with confidence	Bareilly	Salmonella ser. Infantis
0829 5111	Bareilly	Bareilly	Bareilly	Salmonella ser. Bareilly
0922 5132	Javiana	Javiana 100%	Could not be identified with confidence	Salmonella ser. Javiana
0924 5034	Newport	Newport 100%	Bardo/Newport	Salmonella ser. Newport/Bardo
0829 5077	Newport	Could not be identified with confidence	Could not be identified with confidence	Could not be identified with confidence
0829 5079	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
0829 5140	Newport	Newport	Newport/Bardo or Larochelle	Salmonella ser. Newport/Bardo
0922 5033	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
0922 5080	Newport	Newport	Bardo/Newport	Salmonella ser. Newport
0922 5097	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
0922 5103	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
0922 5141	Newport	Newport	Newport/Bardo, or Larochelle	Salmonella ser. Newport
0924 5032	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
0924 5036	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
0924 5057	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
0924 5059	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
0924 5060	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
0924 5061	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
0924 5097	Newport	Newport	Bardo/Newport	Salmonella ser. Newport/Bardo
0829 5148	Typhimurium	Typhiumurium	Typhimurium	Salmonella ser. Typhimurium
0922 5120	Typhimurium	Typhimurium	Typhimurium	Salmonella ser. Typhimurium
0922 5121	Typhimurium	Typhimurium	Typhimurium	Salmonella ser. Typhimurium / 4,[5], 12:i:-
0922 5129	Typhimurium	Typhimurium	Likely Typhimurium	Salmonella ser. Typhimurium / 4,[5], 12:i:-
0924 5079	Typhimurium	Typhimurium	Typhimurium	Salmonella ser. Typhimurium
0924 5119	Typhimurium	Typhimurium	Typhimurium	Salmonella ser. Typhimurium
0924 5139	Typhimurium	Typhimurium	Likely Typhimurium	Salmonella ser. Typhimurium

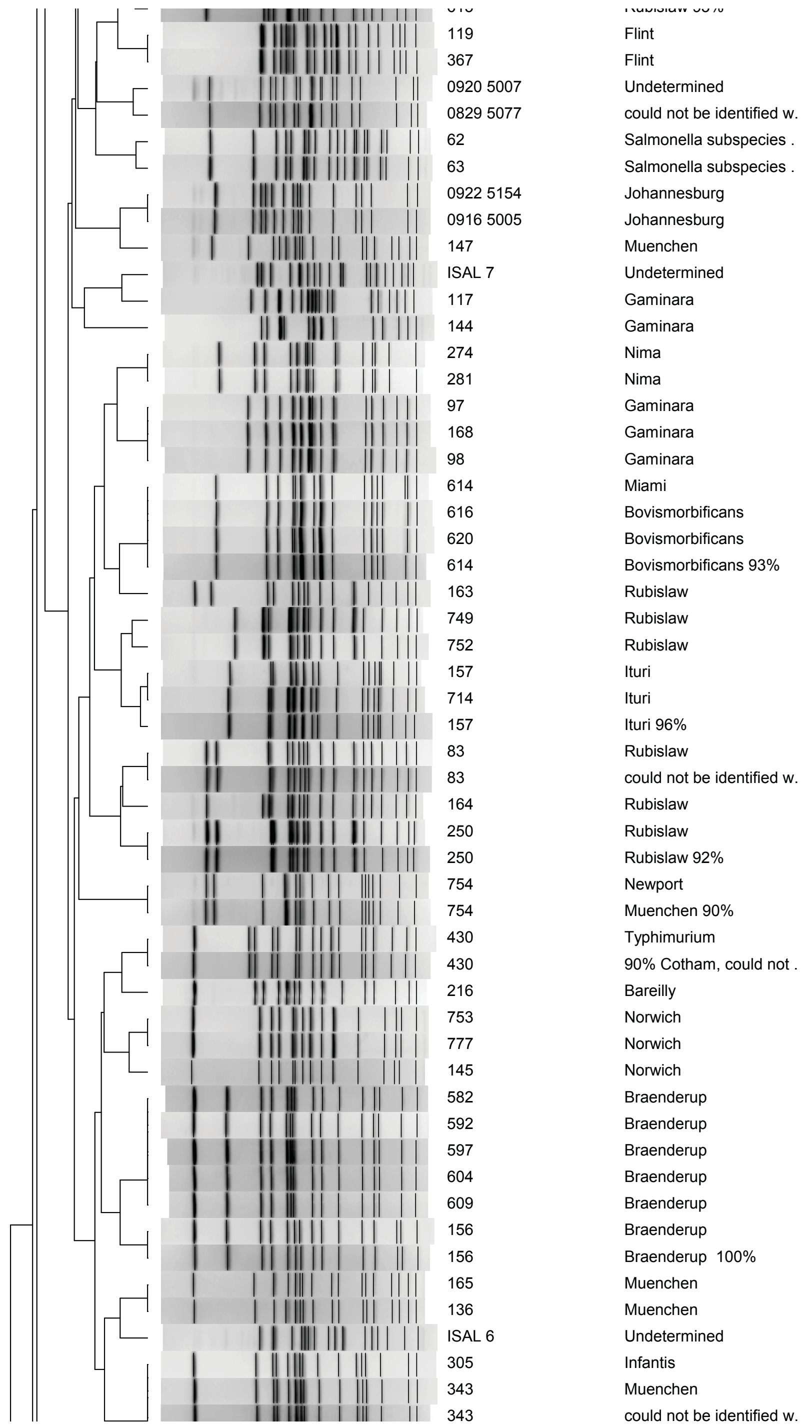
Appendix 1. 6 PFGE dendrogram for comparison of patterns for similarities of all isolates - Central Florida surface water, food processing facility, and fresh produce outbreak (Page 1 of 4).



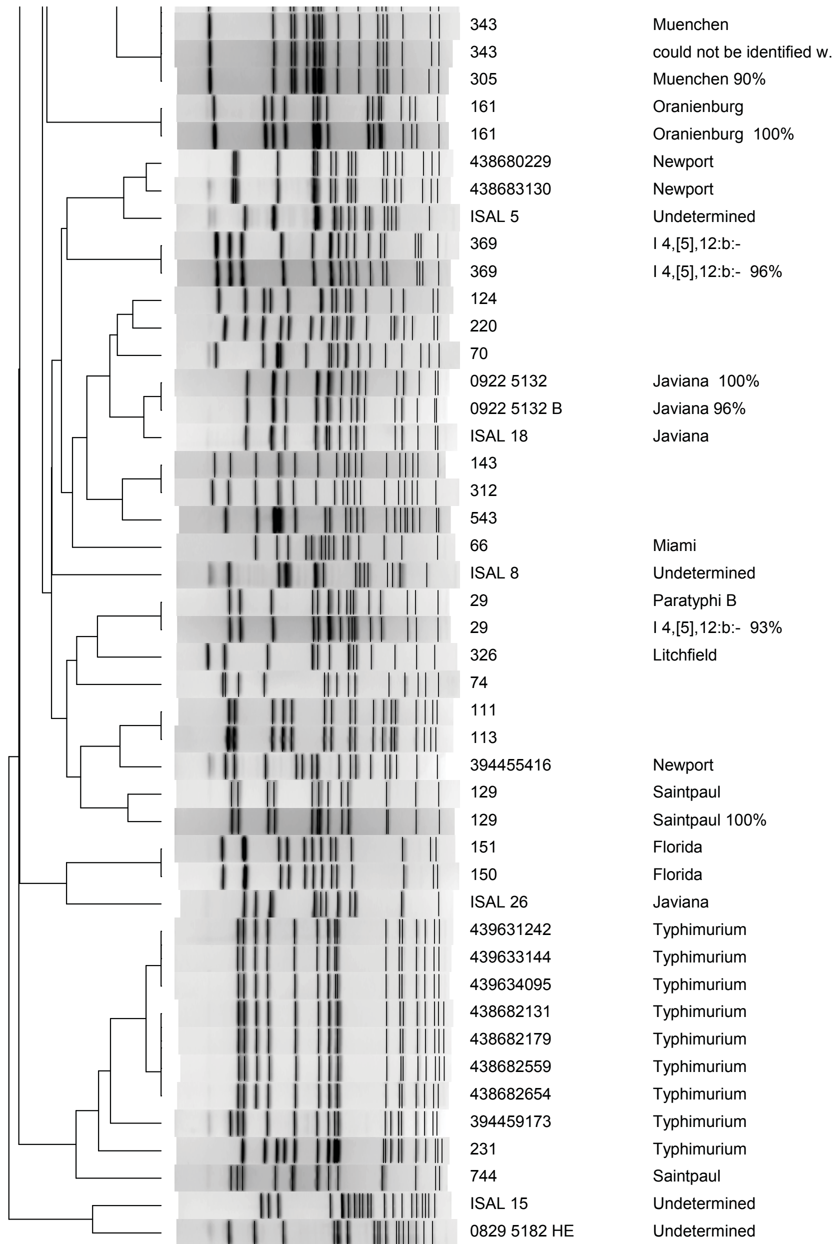
Appendix 1. 7 PFGE dendrogram for comparison of patterns for similarities of all isolates - Central Florida surface water, food processing facility, and fresh produce outbreak (Page 2 of 4).



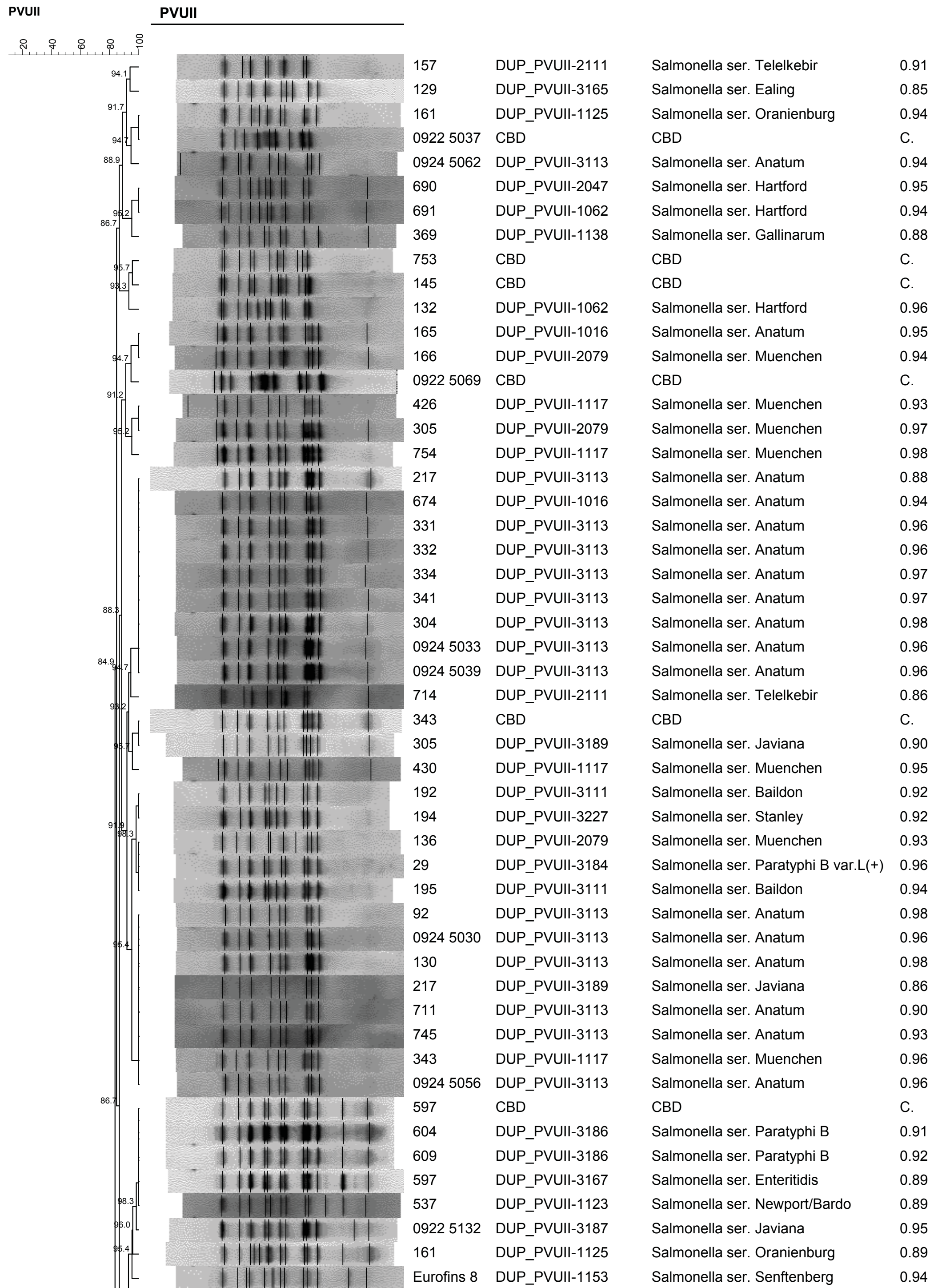
Appendix 1. 8 PFGE dendrogram for comparison of patterns for similarities of all isolates - Central Florida surface water, food processing facility, and fresh produce outbreak (Page 3 of 4).



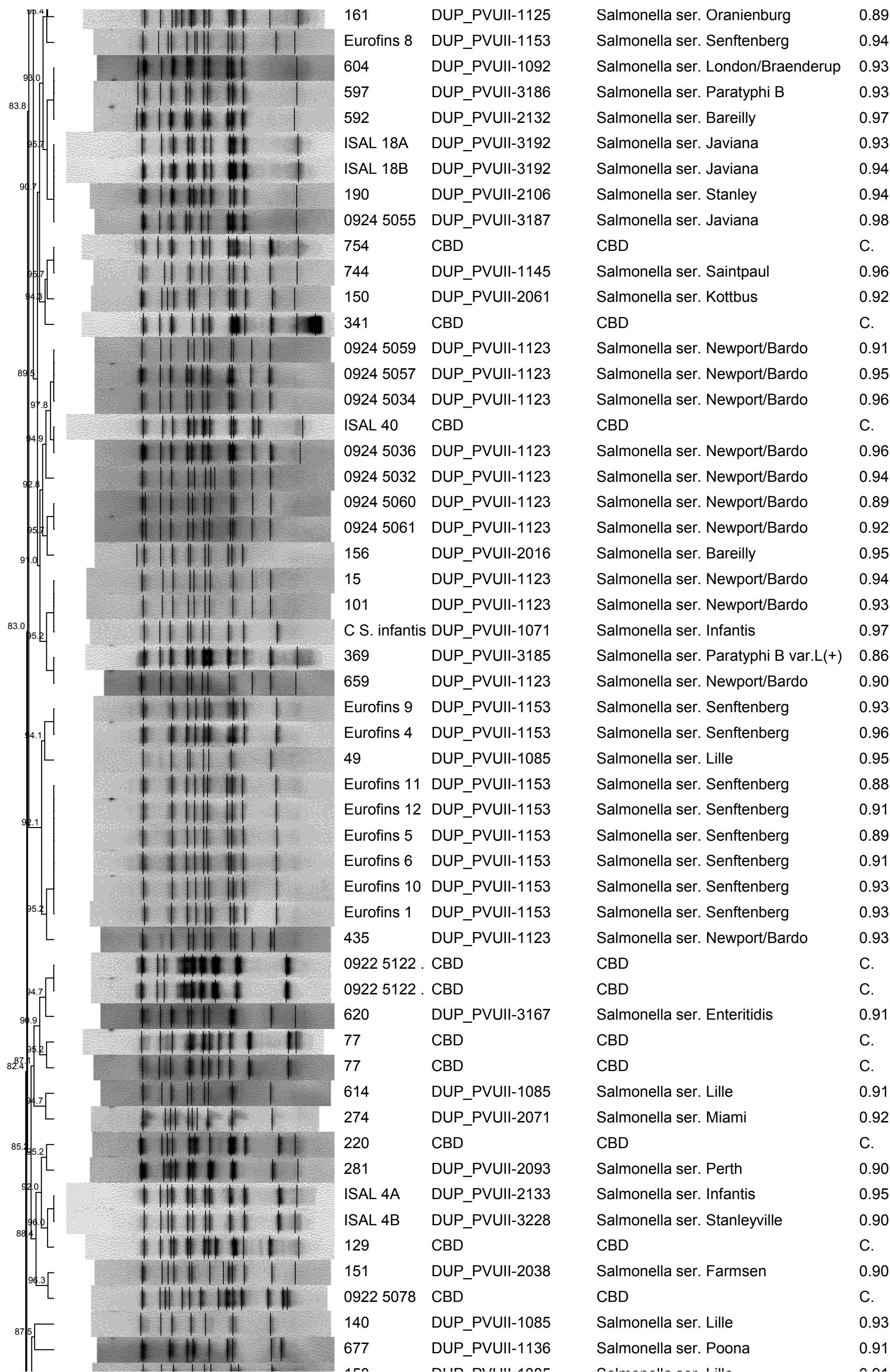
Appendix 1. 9 PFGE dendrogram for comparison of patterns for similarities of all isolates - Central Florida surface water, food processing facility, and fresh produce outbreak (Page 4 of 4).



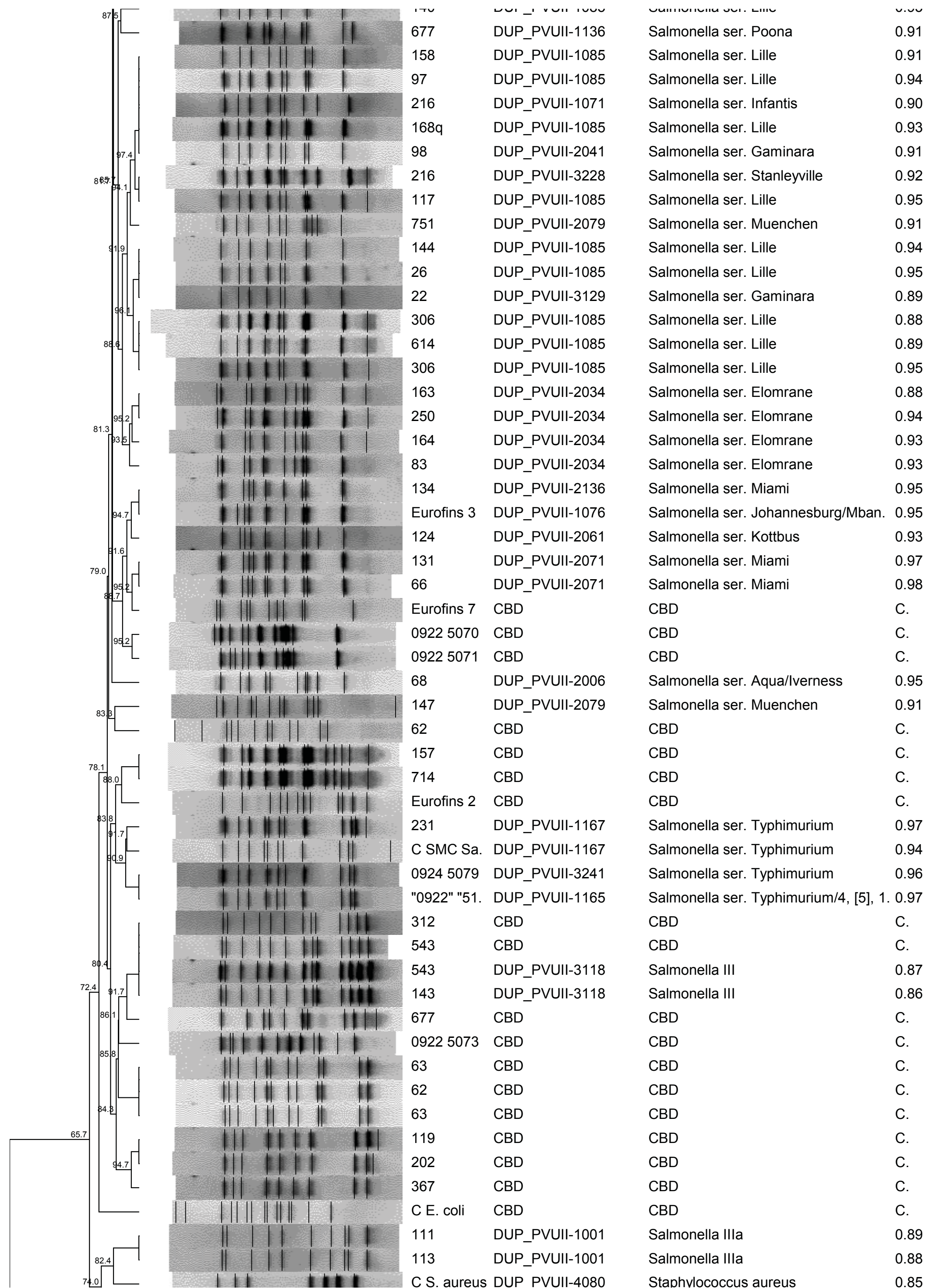
Appendix 1. 10 Automated RiboPrinter ® dendrogram for comparison of patterns for similarities of all isolates -Central Florida surface water, food processing facility, and fresh produce outbreak (Page 1 of 4).



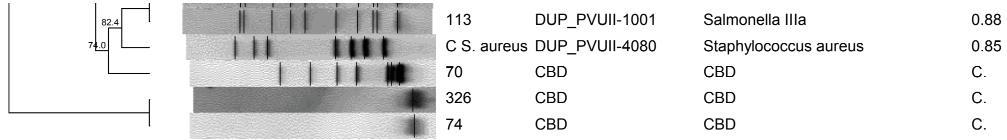
Appendix 1. 11 Automated RiboPrinter ® dendrogram for comparison of patterns for similarities of all isolates -Central Florida surface water, food processing facility, and fresh produce outbreak (Page 2 of 4).



Appendix 1. 12 Automated RiboPrinter ® dendrogram for comparison of patterns for similarities of all isolates -Central Florida surface water, food processing facility, and fresh produce outbreak (Page 3 of 4).



Appendix 1. 13 Automated RiboPrinter ® dendrogram for comparison of patterns for similarities of all isolates -Central Florida surface water, food processing facility, and fresh produce outbreak (Page 4 of 4).



Appendix 1. 14 R Foundation for Statistical Computing (R version 2.15.1) output for greenhouse plant portion samples (Page 1 of 3).

```
R version 2.15.1 (2012-06-22) -- "Roasted Marshmallows"
Copyright (C) 2012 The R Foundation for Statistical Computing
ISBN 3-900051-07-0
Platform: i386-apple-darwin9.8.0/i386 (32-bit)

R is free software and comes with ABSOLUTELY NO WARRANTY.
You are welcome to redistribute it under certain conditions.
Type 'license()' or 'licence()' for distribution details.

  Natural language support but running in an English locale

R is a collaborative project with many contributors.
Type 'contributors()' for more information and
'citation()' on how to cite R or R packages in publications.

Type 'demo()' for some demos, 'help()' for on-line help, or
'help.start()' for an HTML browser interface to help.
Type 'q()' to quit R.

[R.app GUI 1.52 (6188) i386-apple-darwin9.8.0]

[Workspace restored from /Users/swimmer5/.RData]
[History restored from /Users/swimmer5/.Rapp.history]

> testor = rbind(c(0,1),c(85,79))
> testor
     [,1] [,2]
[1,]    0    1
[2,]   85   79
> chi2=chisq.test(testor,correct=F)
Warning message:
In chisq.test(testor, correct = F) :
  Chi-squared approximation may be incorrect
> chi2

	Pearson's Chi-squared test

data:  testor
X-squared = 1.069, df = 1, p-value = 0.3012

> testor = rbind(c(0,10),c(16,23))
> testor
     [,1] [,2]
[1,]    0   10
[2,]   16   23
> chi2=chisq.test(testor,correct=F)
Warning message:
In chisq.test(testor, correct = F) :
  Chi-squared approximation may be incorrect
```

Appendix 1. 15 R Foundation for Statistical Computing (R version 2.15.1) output for greenhouse plant portion samples (Page 2 of 3).

```
> chi2

        Pearson's Chi-squared test

data:  testor
X-squared = 6.0917, df = 1, p-value = 0.01358

> testor = rbind(c(0,0),c(97,101))
> testor
     [,1] [,2]
[1,]    0    0
[2,]   97  101
> chi2=chisq.test(testor,correct=F)
Warning message:
In chisq.test(testor, correct = F) :
  Chi-squared approximation may be incorrect
> chi2

        Pearson's Chi-squared test

data:  testor
X-squared = NaN, df = 1, p-value = NA

> testor = rbind(c(0,0),c(112,218))
> testor
     [,1] [,2]
[1,]    0    0
[2,]  112  218
> chi2=chisq.test(testor,correct=F)
Warning message:
In chisq.test(testor, correct = F) :
  Chi-squared approximation may be incorrect
> chi2

        Pearson's Chi-squared test

data:  testor
X-squared = NaN, df = 1, p-value = NA

> testor = rbind(c(0,1),c(112,217))
> testor
     [,1] [,2]
[1,]    0    1
[2,]  112  217
> chi2=chisq.test(testor,correct=F)
Warning message:
In chisq.test(testor, correct = F) :
  Chi-squared approximation may be incorrect
> chi2
```

Appendix 1. 16 R Foundation for Statistical Computing (R version 2.15.1) output for greenhouse plant portion samples (Page 3 of 3).

```
        Pearson's Chi-squared test

data:  testor
X-squared = 0.5153, df = 1, p-value = 0.4728

>
```

Appendix 1. 17 Statistical Analysis Software Version 9.1 (SAS Institute) output for *E. coli* O157:H7 and concentration via Disposable Inline Filter (DIF).

Time	Conc	Effect	Filter	Filter	Estimate	StdErr	DF	tValue	Probt
0h	0	Filter	POST	PRE	-0.33333	0.666667	2	-0.5	0.666667
0h	1	Filter	POST	PRE	0.333333	0.333333	2	1	0.42265
0h	10	Filter	POST	PRE	2.333333	0.745356	2	3.130495	0.088678
0h	100	Filter	POST	PRE	1	0.57735	2	1.732051	0.225403
12h	0	Filter	POST	PRE	7	6.027714	2	1.161303	0.365382
12h	1	Filter	POST	PRE	38	37	2	1.027027	0.412387
12h	10	Filter	POST	PRE	117.3333	96.2312	2	1.219286	0.347019
12h	100	Filter	POST	PRE	1567.333	381.7811	2	4.10532	0.054527
18h	0	Filter	POST	PRE	3.666667	5.238745	2	0.699913	0.556437
18h	1	Filter	POST	PRE	35.66667	34.67147	2	1.028703	0.411759
18h	10	Filter	POST	PRE	165	144.5095	2	1.141793	0.371815
18h	100	Filter	POST	PRE	1466.333	352.199	2	4.163366	0.053135
8h	0	Filter	POST	PRE	3	2.309401	2	1.299038	0.323519
8h	1	Filter	POST	PRE	49	48.50086	2	1.010291	0.418709
8h	10	Filter	POST	PRE	100.3333	91.63393	2	1.094936	0.387805
8h	100	Filter	POST	PRE	1100	566.9544	2	1.940191	0.191892

Appendix 1. 18 Statistical Analysis Software Version 9.1 (SAS Institute) output for *E. coli* O157:H7 and concentration via Modified Moore Swab (MMS).

Time	Conc	Effect	Filter	Filter	Estimate	StdErr	DF	tValue	Probt
0h	0	Filter	POST	PRE	16	15.01111	2	1.065877	0.398116
0h	10	Filter	POST	PRE	4.666667	3.711843	2	1.257237	0.335589
0h	100	Filter	POST	PRE	0.666667	0.666667	2	1	0.42265
0h	1000	Filter	POST	PRE	3.7E-17	0.471405	2	7.85E-17	1
12h	0	Filter	POST	PRE	100.6667	97.69397	2	1.030429	0.411115
12h	10	Filter	POST	PRE	30.66667	17.05221	2	1.798399	0.213934
12h	100	Filter	POST	PRE	109	9.539392	2	11.4263	0.007572
12h	1000	Filter	POST	PRE	404.3333	18.4421	2	21.92447	0.002074
18h	0	Filter	POST	PRE	113.6667	110.7043	2	1.026759	0.412487
18h	10	Filter	POST	PRE	35.66667	21.58961	2	1.652029	0.240332
18h	100	Filter	POST	PRE	104.6667	4.642796	2	22.54389	0.001962
18h	1000	Filter	POST	PRE	252.3333	29.78628	2	8.471463	0.01365
8h	0	Filter	POST	PRE	93.66667	91.68303	2	1.021636	0.414412
8h	10	Filter	POST	PRE	28	17.47379	2	1.6024	0.250239
8h	100	Filter	POST	PRE	29	6.658328	2	4.355448	0.048882
8h	1000	Filter	POST	PRE	136.3333	41.63465	2	3.274516	0.08196

Appendix 1. 19 Statistical Analysis Software Version 9.1 (SAS Institute) output for *Salmonella* and concentration via Disposable Inline Filter (DIF).

Time	Conc	Effect	Filter	Filter	Estimate	StdErr	DF	tValue	Probt
0h	0	Filter	POST	PRE	70.33333	33.06727	2	2.126977	0.167269
0h	1	Filter	POST	PRE	69.33333	8.034647	2	8.629294	0.013165
0h	10	Filter	POST	PRE	43.66667	20.33333	2	2.147541	0.164825
0h	100	Filter	POST	PRE	42	15.8605	2	2.648088	0.11791
12h	0	Filter	POST	PRE	69	31.54714	2	2.187203	0.160249
12h	1	Filter	POST	PRE	100	29.85521	2	3.3495	0.078749
12h	10	Filter	POST	PRE	6612	2529.648	2	2.613802	0.120483
12h	100	Filter	POST	PRE	10236	742.0087	2	13.79499	0.005214
18h	0	Filter	POST	PRE	84	48.95236	2	1.715954	0.228307
18h	1	Filter	POST	PRE	114.3333	38.10658	2	3.000357	0.095446
18h	10	Filter	POST	PRE	9969.333	683.5543	2	14.58455	0.004668
18h	100	Filter	POST	PRE	10602	268.6826	2	39.4592	0.000642
8h	0	Filter	POST	PRE	78.66667	34.22637	2	2.298423	0.148309
8h	1	Filter	POST	PRE	96	30.56687	2	3.140656	0.088178
8h	10	Filter	POST	PRE	210	84.23974	2	2.492885	0.130214
8h	100	Filter	POST	PRE	255.6667	85.27863	2	2.998016	0.095575

Appendix 1. 20 Statistical Analysis Software Version 9.1 (SAS Institute) output for *Salmonella* and concentration via Modified Moore Swab (MMS).

Time	Conc	Effect	Filter	Filter	Estimate	StdErr	DF	tValue	Probt
0h	0	Filter	POST	PRE	9	2.981424	2	3.018692	0.094449
0h	1	Filter	POST	PRE	10	8.082904	2	1.237179	0.341573
0h	10	Filter	POST	PRE	6.666667	5.456902	2	1.221694	0.34628
0h	100	Filter	POST	PRE	16	7.81025	2	2.04859	0.177049
12h	0	Filter	POST	PRE	25	11.32353	2	2.207793	0.157941
12h	1	Filter	POST	PRE	15	3.21455	2	4.666283	0.042986
12h	10	Filter	POST	PRE	229	69.35897	2	3.301664	0.080776
12h	100	Filter	POST	PRE	2835.333	2033.36	2	1.394408	0.297897
18h	0	Filter	POST	PRE	34.66667	14.49521	2	2.391595	0.139231
18h	1	Filter	POST	PRE	43	19.43365	2	2.212657	0.157402
18h	10	Filter	POST	PRE	6976.667	3204.507	2	2.177142	0.161393
18h	100	Filter	POST	PRE	7187.667	3190.721	2	2.252678	0.153066
8h	0	Filter	POST	PRE	29.33333	20.38518	2	1.438954	0.286789
8h	1	Filter	POST	PRE	9	2.516611	2	3.576237	0.070071
8h	10	Filter	POST	PRE	15.33333	6.489307	2	2.362861	0.141946
8h	100	Filter	POST	PRE	17	6.027714	2	2.820306	0.106088