Association of *Salmonella enterica* serotype Newport with Tomato Plants through Irrigation Water, Grown under Controlled Environmental Conditions

By

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ABSTRACT:

Tomato fruit have been associated with numerous outbreaks of salmonellosis in recent years. Trace back suggests tomato fruit may become contaminated during pre-harvest, however exact routes are unclear. The objective of this study was to determine the potential for *Salmonella enterica* serotype Newport to be associated with the roots, leaves, stems, and ultimately fruit of red round tomato plants through contaminated irrigation water, at various stages of plant development, when grown under controlled environmental conditions. Tomato plants were individually root irrigated with 250 or 350 ml (depending on growth stage) of 7 log CFU/ml *S*. Newport contaminated irrigation water every seven days. Presence of the pathogen in plant tissue was evaluated at five growth stages; 14 days post transplant, early fruit, mid fruit, full fruit, and terminal stages. At each stage, roots, stems, leaves, and two tomato fruit, if present, from four *S*. Newport and four water irrigated (negative control) tomato plants were sampled for *S*. Newport contamination. Association of *S*. Newport was detected in tomato roots and stems using both conventional plating and molecular techniques. Twenty-four samples were confirmed positive for *S. enterica* using PCR. Sixty-five percent of the roots, 40% of the stems, 5% of the leaves and 5% of the fruit sampled were confirmed to contain *S. enterica*. Overall, there was significant difference in the presence of *S.* Newport according to tissue sampled (roots > stems > leaves and fruit) ($P > 0.05$). There was no correlation between growth stage and presence of *S*. Newport in tissues ($P > 0.05$). Ultimately, irrigation with *S*. Newport has a low probability of contaminating tomato fruit.

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DEDICATION

I dedicate this work to my parents, Morse and Debby Hintz, for teaching me that quitting is never an option!

Attribution

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Renee R. Boyer- Ph.D. (Department of Food Science and Technology, Virginia Tech) is the primary Advisor and Committee Chair. Dr. Boyer provided constant assistance and guidance throughout this research work. Furthermore, Dr. Boyer also provided funds for all of the supplies involved with this project.

Chapter 3: Association of *Salmonella enterica* serotype Newport with Tomato Plants Grown under Controlled Environmental Conditions

Monica A. Ponder- Ph.D. (Department of Food Science and Technology, Virginia Tech) was a member of the author's committee. Her mentorship and background in molecular biology significantly contributed to this project.

Dr. Steven Rideout- Ph.D. (Department of Plant Pathology and Weed Science, Virginia Tech) was a member of the author's committee. His assistance and background in applied plant pathology greatly contributed to this work.

Robert C. Williams- Ph.D. (Department of Food Science and Technology, Virginia Tech) was a member of the author's committee. His mentorship and background in produce food safety greatly aided this research work.

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Appendix A: Preliminary data and trouble shooting procedures for study: Association of *Salmonella enterica* serotype Newport with Tomato Plants Grown Under Controlled Environmental Conditions

Monica A. Ponder- Ph.D. (Department of Food Science and Technology, Virginia Tech) was a member of the author's committee. Her mentorship and background in molecular biology significantly contributed to this project.

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CHAPTER 1:
INTRODUCTION AND JUSTIFICATION:

Fresh produce consumption has increased dramatically over the last two decades. From 1982 to 1997, per capita consumption of fresh produce increased 32% from 91.6 to 121.1 kg per annum *(3)*. The consumption of fresh tomatoes (*Lycopersicon esculentum* L.) has increased in the United States because of their consumption in salads and sandwiches, growing attention to health and nutrition, and increasing popularity of vegetarian and vegan diets *(12)*. The United States is one of the leading producers of tomatoes in the world *(9)*. Approximately 5 billion pounds of tomatoes are eaten annually in the United States *(1)*. Farm value of tomato production is $1.3 billion and the retail value of fresh market tomatoes is estimated to be over $4 billion *(9)*. Since the early 1990s, the annual per capita use of fresh-market tomatoes has dramatically increased because of domestic greenhouses and hydroponic system expansion while the use of processed tomatoes has leveled off *(10)*.

An increase in the number of food-borne illnesses linked to fresh produce is coupled with the increase in produce consumption *(3)*. The safety of fresh-market tomatoes and their potential to cause food-borne illness is of growing concern. From 1998 to 2006, 71 produce related outbreaks have been reported, of which 12 were linked to tomatoes *(1, 2)*. Three *Salmonella enterica* serovar Newport outbreaks were linked to tomatoes grown on the Eastern Shore of Virginia *(6)*. *S.* Newport accounts for 42% of *Salmonella* outbreaks in Virginia *(6)*. Without a definitive cause of the outbreaks, the tomato industry seeks possible solutions to prevent and reduce *Salmonella* contamination.

Some of the most common reservoirs for *S. enterica* are birds, amphibians, and reptiles. Tomatoes, and other produce, are grown in areas where those animals are found naturally. Sporadic contamination of the tomato fruit could occur through close proximity to these natural reservoirs.

Microorganisms can enter tomato plants through various pathways. Potential *Salmonella* entry routes for tomato plants could include roots, flowers, or into the fruit via the stem scar or cracks in the cuticle. Conflicting research exists regarding whether entry into the plant tissues results in contaminated fruit. When Jablasone *et al.* applied

contaminated water directly onto the soil of pots containing cultivar Cherry Gold tomatoes, *S. enterica* Enteriditis was not recovered from the stems, leaves, or fruits of tomato plants *(7)*. Additional *S. enterica* Montevideo irrigation studies performed on greenhouse tomatoes, found no contaminated tomatoes, but samples of roots exposed to 4 or more irrigation events (one every 14 days) were positive *(11)*.

Inoculation of *Salmonella* into aerial plant tissues resulted in contaminated tomatoes. Guo *et al.* showed tomato plants inoculated with a cocktail of five *S. enterica* serotypes (Montevideo, Michigan, Poona, Harford, and Enteriditis), either inoculated onto tomato flowers or injected into the stem, produced 37% of fruit positive for *Salmonella (5)*. *S. enterica* is capable of survival on and in tomato fruit *(8)*. Lin *et al.* found that *S.* Montevideo is able to survive on tomatoes' stems and cracks *(8)*.

Tomato fruit could also become contaminated during post-harvest processing. After harvest, tomato fruits are commonly deposited in a chlorinated water filled dump tank and transported through a flume system. Both the dump tank and flume water are typically treated with chlorine, however the chlorination treatments are completed primarily to maintain water quality *(13)*. Treatment of tomato fruit with chlorinated water cannot completely decontaminate microbes within the interior of the fruit, thus making it difficult for this treatment to ensure tomato safety *(13)*. Currently, no effective post-harvest kill step exists for removing *S. enterica* from tomato fruits. Thus, prevention of *S. enterica* introduction into tomato plants is essential for preventing future outbreaks.

In 2007, the United States Food and Drug Administration (FDA) implemented a multiyear initiative aimed at reducing tomato related illnesses *(4)*. Goals of this initiative include identifying production practices or conditions, which may lead to tomato contamination, as well as facilitating and promoting tomato safety research *(4)*. The increasing number of outbreaks has raised concerns about the pre-harvest *S. enterica* colonization of tomatoes *(4)*. This study hopes to mimic natural growing conditions as closely as possible while continuing the FDA's efforts by determining whether tomato plants irrigated with *S.* Newport have the potential to uptake the organism into roots, stems, leaves, or fruit of the plant resulting in contaminated tomatoes. Understanding whether or not this phenomenon occurs may provide more complete understanding of *Salmonella* entry points and help determine mitigation strategies, which could prevent

tomato related Salmonellosis outbreaks in the future.

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Chapter 2: Literature Review

Salmonella enterica:

S. enterica is a gram negative, rod shaped, motile bacterium that is a leading cause of food-borne illness in the United States *(35)*. There are over 1.3 million cases and 600 deaths of human salmonellosis annually, in the United States, with the majority going unreported *(23)*. To cause disease, *S. enterica* cells must have sufficient concentration to overcome host defenses. The concentration required for disease is affected by the physiological state of the bacteria, the matrix of the bacteria ingested, the context of the meal, and the degree of the hosts' resistance *(23)*. Salmonellosis occurs when *S. enterica* enters the small intestine and the lining becomes inflamed. Acute symptoms of salmonellosis, such as nausea, vomiting, abdominal cramps, diarrhea, and headaches, can occur within 6 to 48 hours of ingesting *Salmonella* contaminated food *(35)*. Acute symptoms usually last between one and two days but as time elapses, the severity can increase and arthritic symptoms, known as Reiter's syndrome, may result *(35)*. Most people recover with bed rest and fluid balance. While most individuals recover from food borne illness without complications or need of medical attention, some individuals, such as infants, elderly, and those who may be immuno-compromised may suffer complications, and even death *(35)*. Young children are approximately five times more likely to contract salmonellosis then adults *(7)*. In the year after a *S. enterica* infection, mortality rate increases three times compared to those of controls *(23)*. *S. enterica* serovars have enormous economic impact and are the most important food-borne pathogen, in terms of deaths caused *(23)*.

Food-borne Outbreaks Associated with Fresh Produce:

Previously believed to only colonize intestinal habitats, outbreaks associated with enteric pathogens in produce continues to become diverse, steadily increasing, and a new vehicle for zoonotic disease *(5)*. In recent years, the produce industry has undergone changes that might factor into produce related outbreaks. The produce industry has become more centralized in production and items are distributed over longer distances, including a rise in imported produce *(46)*. Consumers have also changed their habits by increasing consumption of fresh fruits and vegetables and meals eaten outside the home, especially

salad bars *(46)*. Despite improved surveillance and pathogen tracking methods, pathogens are emerging with low infectious dose and the size of the at-risk population is increasing *(46)*. With new scientific evidence for the growth and survival of enteric pathogens on fresh produce, risk factors that contribute to produce related outbreaks must be identified so mitigation strategies can be developed.

Tomatoes:

Global production of tomatoes, fresh and processed, has increased 300% in the last four decades *(9)*. The cultivated tomato is a perennial diploid dicotyledon and most fresh market field varieties have indeterminate growth *(9)*. Tomato plants are an annual warm season crop, but are sensitive to temperature extremes, especially below 13°C and above 35°C. Tomatoes prefer deep, well-drained soils with a mixture of sand, clay, silt, and organic matter. The soil should be slightly acidic with a pH of 6.2 to 6.8 and tomatoes respond well to fertilizers with high phosphorus content *(9)*. Staking tomato plants helps reduce contact of the fruit with the soil and is a commercially followed practice *(9)*. Tomatoes are climacteric fruits and have increased respiration and ethylene biosynthesis during ripening *(6)*.

Tomatoes are commonly used as a model crop because of their short life cycle and easy manipulation. However, certain environmental conditions must be present for tomato plant growth. Soil salinity helps increase both fruit quality and soluble solids, but in high levels severe wilting and permanent damage is likely *(39)*. By watering tomatoes frequently, the plants experience less stress and salinity extremes *(39)*. When soils have excessive moisture content, poor roots develop with increased potential for root disease *(39)*. Field grown tomatoes are mildly tolerant of acidic soil conditions but if the soil is too acidic, nutrients such as calcium, phosphorus, magnesium, and molybdenum are most likely deficient *(39)*. As air temperature increases, water uptake increases independently of nutrient uptake, leading to a higher concentration of nutrients in the soil and increases soil salinity *(39)*. Calcium uptake is also dependent on transpiration rate. Calcium moves through the xylem and if not properly transported, blossom end rot and other stress conditions may result *(39)*.

Tomatoes produce a variety of secondary metabolites that protect against damage from fungi, bacteria, viruses, and insects *(16)*. A major metabolite, tomatine has beneficial antibiotic properties in tomato plants. Tomatine inhibits the growth of *Lactobacilli* species and reduces the prevalence of insects and snails on the plant *(16)*. Tomatine consists of two glycoalkaloids; α- tomatine and dehydrotomatine. Immature green tomatoes contain α- tomatine, which is involved in host- plant resistance. α- tomatine possesses antibiotic properties against fungi and the pathogenic gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus (25)*. However, α- tomatine has no significant effect on the gram negative bacteria *Escherichia coli (25)*. It is unknown how *Salmonella* species respond to α- tomatine *(16)*. Additionally, α- tomatine assists tomato plant disease resistance *(16)*. Glycoalkaloids allows the tomato plant to resist pathogens by binding to cholesterol and disrupting cellular membranes *(16)*. The glycoalkaloid concentration degrades as the fruit matures because the plant produces enzymes that degrade glycoalkaloids *(16)*. When tomato fruits are ingested, the molecule retains its activity in animals and humans, producing beneficial dietary properties *(16)*. Excessive sunlight is a stress factor for the tomato plant and induces the synthesis of anti-feeding compounds to protect the plant from insects *(16)*.

Tomato Production:

Tomato varieties are selected based on the channels through which the tomato fruit will be marketed. Indeterminate varieties produce fruit for an extended time period and are preferred for fresh tomato markets *(42)*. These varieties are hand harvested to maximize the tomato fruit yield *(42)*. Fresh market tomatoes are picked mature green through red ripe stages *(42)*. Mature green fruit are more likely to survive post-harvest stress and have an increased shelf life when compared to red ripe tomatoes *(42)*. Immediately after harvest, the fruit is transferred to bins and transported to the packinghouse for culling, sorting, and packaging. Fresh market tomatoes typically have sufficient time for water loss and pathogenic infections, as the fruit may sit after harvest *(42)*.

Tomatoes used for processing are typically determinate varieties. Determinate varieties are mechanically harvested when 90% of the fruit is ripe *(42)*. Ethylene releasing chemicals may be applied to maximize the ripe fruit percentage *(42)*. The entire plant is

mechanically lifted from the soil and the fruit is separated into field bins. *(42)*. After harvest they are immediately transported to the processing facility, to preserve fruit quality *(42)*. After harvest, the fruit temperature should be cooled as quickly as possible by shading and forced air-cooling *(42)*.

After harvest, tomato fruit used for processing and fresh market varieties are processed, packaged, and distributed. At the packing shed, tomato fruit are dumped into warm water to remove any loose dirt or debris. Warm water, ten degrees above pulp temperature, is used because cold water will cool the gases inside the fruit and water can enter the fruit through the stem scar *(42)*. Infiltration of water into the tomato is undesirable because the water could carry chemicals or biological pathogens, including *S. enterica*, directly to internal fruit tissues. Bartz showed that water infiltration into tomato fruit was positively correlated to both the flume water temperature and the depth of fruit submersion in the water *(2)*. A negative temperature differential is created when cold water, 15ºC cooler then the fruit, is used *(2, 50)*. Water uptake occurred in the vascular area beneath the stem scar *(2, 3)*. The fruit are culled, sorted, graded, and packed in boxes with adequate ventilation *(42)*. Ripening can occur naturally, but is generally induced by ethylene, while in the packed boxes either at the packinghouse or distribution center *(42)*. After the fruit is ripe, it is distributed to markets and retail store, then ready to be purchased for further processing or consumption.

Pre-harvest Contamination Routes:

Salmonella species can be distributed across a wide variety of farm types or locations. Rodriguez *et al.* collected on-farm samples, field produce, water, equipment, and soil, from 18 farms across five states over a 24-month period to evaluate the distribution of *S. enterica* species in farm environment *(41)*. After extensive sampling, 4.7% of samples were *Salmonella* positive, finding *S.* Anatum, *S.* Arizoniae and *S.* Javiana most often *(41)*. *Salmonella* can also be found in greenhouse environments. Orozco *et al.* sampled a Mexican hydroponic tomato greenhouse and also found *S. enterica* positive samples in puddles, soil, cleaning cloths, sponges, and tomato fruit (2.8%) showed *S. enterica* contamination *(36)*. Orozco *et al.* conducted a second environmental sampling where they focused on the sources of the previous *Salmonella* contamination *(37)*. Overall, the number

of *Salmonella* positive samples decreased over time but tended to increase following two environmental disturbances *(37)*. Flood runoff entered the greenhouse and after the water drained, *S. enterica* Newport was seen in a variety of samples including tomatoes *(37)*. Wildlife also entered the greenhouse and after this incident, *S.* Montevideo positive samples increased *(37)*. Both incidents show that a hydroponic greenhouse can become contaminated despite physical barriers *(37)*.

Soil:

Soil is another potential tomato contamination route. Factors affecting the survival of *S. enterica* in the soil include the initial concentration of *S. enterica*, temperature, frost, moisture content, humidity, sunlight, salinity, soil texture, organic matter content, and other microorganisms *(26)*. *S. enterica* serovars have been reported to survive in soil amended with sewage sludge up to 968 days *(29)*. If the soil is fertilized with cattle slurry or animal feces, *Salmonella* can survive up to 300 or 259 days, respectively *(29)*. Others found that in moist soil, with or without sewage sludge, *Salmonella* can survive greater then 45 days *(20)*.

Several studies have shown human food-borne pathogens directly applied to plants survive for shorter periods of time than those applied to soils. Barak *et al.* showed that *S. enterica* contaminated soil can contaminate a tomato's phyllosphere *(1)*. *S. enterica* was capable of surviving in soil and contaminating a tomato's phyllosphere six weeks after *S. enterica* was inoculated in the soil *(1)*. Contact with contaminated soil, increases the *S. enterica* population by 2.5log CFU per tomato fruit during four days of storage at 20 °C *(20)*. After 24 hours of soil contact, *S.* enterica harbored near the tomato fruit skin surface but over time was visible in the stem scar and subsurface tissue *(20)*.

Manure, when used as an agricultural fertilizer, can pose a severe threat to food safety if not properly treated. When enteric food borne pathogens are shed from an infected animal or human through feces, this contamination can then spread to fresh uncooked food products. *S.* Newport specifically, can survive in manure and manure amended soil for greater then 184 days *(49)*. You *et al.* found that contaminated manure, manure amended non-sterilized soil, and manure amended sterilized soil provided an environment for *S.* Newport to survive for 184, 332, and 405 days respectively *(49)*. Over time all samples

decreased according to first order kinetic models *(49)*. Some indigenous microorganisms may antagonize the growth of *S.* Newport leading to prolonged survival in manure amended sterilized soil *(49)*. Compost teas, unheated compost treatment turned into a spray or soil drench, also provide an environment suitable for *Salmonella* to grow *(24)*. Adding commercial supplements to compost teas increased the growth of *S.* Enteriditis by 1 to 3 log CFU/g in both aerated and non-aerated compost teas *(24)*.

Infected tomato plant debris can spread *S. enterica* contamination to a subsequent tomato crop. Barak *et al.* contaminated a tomato plant crop with *S. enterica* and after 30 days mulched the crop and mixed the debris into the soil *(1)*. A second crop was planted and sampled for *Salmonella* contamination *(1)*. If the debris was incorporated 24 hours before planting the seeds, *S. enterica* contamination was seen in both the tomato plant rhizosphere and phyllosphere *(1)*. As the plants grew to three to five true leaf stages, enrichment was needed for *S. enterica* detection *(1)*. When the debris was incorporated seven days before planting the seeds, *S. enterica* contamination was not observed in the phyllosphere and remained below 100 CFU/g in the tomato rhizosphere *(1)*. *S. enterica* soil contamination can be transferred to a subsequent tomato plant crop depending on the time between crop incorporation and the next seeding *(1)*.

Water:

Irrigation water is another potential source of tomato contamination. When manure is applied to land, enteric pathogens are likely to move throughout the soil matrix. Water availability and movement are key elements in enteric pathogen migration *(49)*. The degree of pathogen movement potential dictates the likelihood that the pathogen will reach an irrigation water source or be consumed by livestock *(49)*. Once *S. enterica* enters a water supply, it is more capable of survival in stream bottom sediments than surface water *(22)*. The organism can find appropriate nutrients in the sedimentation by adsorption through the sand and clay *(22)*. Maki *et al.* determined that populations of *S.* Typhimurium decrease when suspended in freshwater particles because the bacterium may not absorb nutrients effectively or remain attached *(33)*.

After *S. enterica* contaminates irrigation water, contamination could become associated with produce. Carrots and radishes were seeded in *S.* Typhimurium irrigated

soil *(26)*. *S. enterica* contamination was detected on carrots and radishes up to 203 and 84 days, respectively *(26)*. Lettuce and parsley, grown under the same conditions were contaminated for 63 and 231 days respectively *(27)*. After irrigating a variety of produce crops with *S. enterica* contaminated water, crops that are prostrate, such as lettuce, were more contaminated then those crops grown above the soil surface, such as tomatoes *(34)*. Edible produce crops that develop prostrate are in direct contact with contaminated water during irrigation. These crops also tend to have higher levels of foliage where contaminants can reside *(34)*.

Produce crops with edible fruits above the soil have less chance for contamination. Jablasone demonstrated this when tomato plants were watered every other day with *S.* Enteritidis contaminated irrigation water to determine if *S.* Enteritidis can be internalized *(28)*. While *Salmonella* remained in the soil, *S.* Enteritidis was not recovered from leaf, stem, and tomato fruit *(28)*. The experimental conditions provided minimal root damage and *S.* Enteritidis was not internalized in the tomato's phyllosphere *(28)*.

Sampling spent irrigation water that has flowed through tomato drip lines may be a good indication of the types of microorganisms in the plants themselves *(11)*. The microorganisms recovered from spent irrigation water are about one log less than the level in the plant *(11)*. So, if pathogens are present at low levels, it is possible they will be missed in the water but present in the plant. Farms using overhead irrigation systems contain an increased frequency and higher bacterial concentration in their produce than furrow irrigated farms *(34)*. Overhead irrigation systems are discouraged by GAPs protocol.

Pesticides do nothing to affect the growth of *S. enterica (18)*. Therefore, if pond water or other non-potable water sources contaminated with *S. enterica* were used as a carrier for pesticides sprayed onto crops, the crops may become directly contaminated with *S. enterica* at concentrations equal to the pond water. The infiltration and persistence of *S. enterica* on produce is directly affected by the quality of water being used on and around the crop.

Survival and Growth of *S.* Newport in and on Various Plant Structures:

S. Newport is capable of internalizing and growing in some plant tissues. When *Arabidopsis thaliano* (thale cress) was inoculated at the roots with *S.* Newport, under hydroponic sterile conditions at 100% humidity, contamination occurred throughout the plant, showing the bacterium is capable of moving on or within the plant when no competitors are present *(8)*. Contamination spread was eliminated when non-motile *S.* Newport was used and decreased as the plant matured *(8)*. Authors hypothesized that indigenous soil epiphytes could compete with *S.* Newport in the model plant system because the *S.* Newport concentration decreased in non-autoclaved soil *(8)*. When co-inoculating *Enterobacter asburiae* and *S.* Newport into the sterile model plant root system, epiphytic growth of *S.* Newport was suppressed *(8)*. Cooley *et al.* also showed that there is a strong correlation between contamination of the plant chaff and the seed *(8)*. Contamination of the seed may occur when contamination spreads from the chaff, flower, silique, or protected seed niches *(8)*. Whether these same *S.* Newport interactions can be shown in tomato plants has yet to be determined.

Research suggests tomato flower contamination can be transferred to green immature and ripened tomato fruit. Five different serovars, *S*. Montevideo, *S*. Michigan, *S*. Poona, *S*. Hartford, and *S*. Enteritidis, were introduced onto tomato flowers as a cocktail inoculation, then the tomato fruit was screened for *Salmonella* contamination *(19)*. *S*. Montevideo was the serovar most frequently recovered on and within tomato fruit followed by *S*. Poona *(19)*. The other serovars were less frequently recovered after flower inoculations *(19)*.

Certain *S. enterica* serovars have adapted to persist on or within intact tomato fruit. Shi *et al.* determined that the majority of *S. enterica* serovars implicated in previous food-borne illness can grow on and within green tomatoes but growth on ripened red tomatoes is serovar dependent *(43)*. Additional studies were conducted by individually adding *Salmonella* serovars, Javiana, Montevideo, Newport, Dublin, Enteritidis, Hadar, Infantis, Typhimurium, and Seftenberg, to tomato flowers *(43)*. All serovars persisted in green fruit with *S*. Montevideo recovered in 90% of green fruit. In mature red fruit, only *S*. Hadar, *S*. Montevideo, and *S.* Newport were adapted for growth *(43)*.

Hand- washing, Health, and Hygiene:

Workers who harvest, package, and handle produce can also serve as a route to transfer pathogens onto fresh produce *(14)*. If the workers do not wash their hands after using the restroom, enteric bacteria can directly contaminate the food they are handing. Workers who are ill also can contaminate the food through gastrointestinal symptoms, infected wounds, or cuts. Educating workers about the risk of pathogens can reduce microbial spread from workers to the fresh produce.

Outbreaks:

S. Newport accounts for four percent of all human *Salmonella* infections globally *(45)*. *S.* Newport is the third most common serotype causing human illness, with an estimated 100,000 people becoming infected each year *(17)*. Outbreaks have resulted from foods of animal and produce origin. *S. enterica* related outbreaks increase in frequency each year in the United States *(7)*. From 1996 to 2006, 71 produce related outbreaks have been reported and 12 of those can be linked to tomatoes (Table 2.1) *(45)*. Over the past ten years, four out of twelve tomato-associated outbreaks have been traced to produce originating from the Eastern Shore of Virginia or Florida (Table 2.1) *(45)*. However, tomato outbreaks have also been traced back to Georgia, South Carolina, Ohio, and California. In 2002, tomatoes grown on the Eastern Shore of Virginia were traced to an outbreak of *S.* Newport that caused 510 patients in 22 states to became ill *(17)*. The strain was recovered from July to October 2002, with 282 confirmed cases of *S.* Newport containing matching PFGE patterns *(17)*. After performing multiple control studies tomatoes from the Eastern Shore of Virginia were implicated in the outbreak *(17)*. In 2005, an outbreak of *S.* Newport was traced back to Eastern Shore of Virginia tomatoes in which, 72 patients in 16 states, mostly in the eastern United States, became ill *(17)*. Trace-back analysis indicated that the contaminated tomatoes ingested were red round varieties *(17)*. Irrigation water in close proximity to the tomato field also tested positive for the strain, however no tomatoes were found positive *(17)*. Investigators were not able to confirm how the water became contaminated *(17)*. In 2006, a third outbreak of *S.* Newport was linked to the Eastern Shore of Virginia's tomato crop *(17)*. All three outbreaks shared the same PFGE pattern without a single shared event to cause the numerous illnesses *(17)*. All three

outbreaks peaked between September and November correlating with the growing season *(17)*.

In the summer of 2004, three separate *Salmonella* outbreaks were detected in the United States due to ingesting Roma tomatoes *(7)*. A chain delicatessen was responsible for a multi-state outbreak where multiple *Salmonella* serotypes were isolated from 429 salmonellosis cases *(7)*. There was a strong association between those individuals that ate the Roma tomatoes and the illness *(7)*. The contaminated pre-sliced tomatoes were purchased from a single processor for all stores in the chain *(7)*. *S.* Braenderup was implicated in the second outbreak after 125 cases were confirmed following individuals ingesting Roma tomatoes *(7)*. *S.* Javiana was responsible for the third outbreak, after seven confirmed cases in Ontario, Canada *(7)*. The individuals consumed Roma tomatoes at the same restaurant *(7)*. All three outbreaks were traced back to a single packinghouse in Florida *(7)*.

Outbreaks of food-borne illness are traced back to the source of contamination by using Pulse Field Gel Electrophoresis (PFGE) *(44)*. The different bacterial strains' PFGE patterns are tracked by PulseNet at the National Molecular Subtyping Network for Food-borne Disease Surveillance, located at the Center for Disease Control and Prevention (CDC) *(44)*. State public health laboratories submit PFGE patterns from *Salmonella* cases and PulseNet compares the patterns to detect national clusters of illness *(44)*. A second epidemiological method is the *Salmonella* Outbreak Detection Algorithm (SODA) *(40)*. SODA uses a computer algorithm to detect *Salmonella* clusters reported to public health laboratories *(40)*. SODA compares any unusual amount of cases to a baseline based upon the average number of illnesses reported over the past five years *(40)*. PulseNet and SODA only have the ability to compare and locate outbreaks in the Unites States because of differences in surveillance systems between different countries *(45)*. Fresh fruits and vegetables are difficult to trace back, after an outbreak is confirmed, because they are perishable and lot numbers and grower identifications are not routinely used or recorded *(12)*.

Recent tomato outbreaks of salmonellosis share many characteristics. They are widely dispersed throughout the United States and in many states only individual patients become infected. This suggests the contamination occurs early in the distribution chain, at

the farm or packing house level, instead of at restaurants *(17)*. The low incidence rates associated with tomato salmonellosis make the trace-back epidemiology tedious. Many patients fail to recall eating tomatoes with other produce therefore it is difficult to conclusively say which food was the cause of the illness. Multiple suppliers, distributors, packers, and re-packers are typically involved in the transportation of tomatoes to the consumer and it is not easy to find the initial source of contamination. Once the produce is deduced as contaminated it is difficult to find a sample for pathogen confirmation because the produce is rarely still available either in the fields or from the consumer. When a restaurant is associated with an outbreak, possible sources of contamination may occur. The restaurant can receive tomatoes from multiple sources and might store or handle them differently, such as mixing different sources together in storage. This could spread contamination through a larger quantity of food and amplify the bacterial growth and severely complicate trace back efforts *(17)*.

Points of Control:

Points of control are necessary to reduce potential *Salmonella* contamination in the food chain. Control of *S. enterica* requires the identification of science based, cost effective strategies to protect all parts of the food chain *(23)*. *S. enterica* frequently changes mechanisms of survival and persistence in both animal and human hosts, leading to difficult control strategies *(23)*. The various points of tomato fruit contamination can occur anywhere from farm to table. Possible sources include irrigation water, runoff water from livestock or produce farms, wash water, handling by field workers and processing employees, contact with contaminated surfaces, fertilizers, and feces of rodents or ruminants *(4)*. It is at these points along the farm to fork continuum that control can be used to mitigate potential contamination events. When tomato fields have poor drainage or heavy rains, the tomato plants are subjected to standing water, which can lead to higher levels of contamination. During drought conditions, the water line in the drainage ditch decreases, causing the living organisms to seek a new source of water, most likely closer to the tomato fields. Maintenance of water quality especially monitoring the chlorine levels, pH, and water temperature in wash tanks is an important point of control *(7)*. Several of the *Salmonella* related outbreaks in tomatoes have been traced to the wash tanks in

packinghouses. Water quality should be adequate for its intended use and if the water quality cannot be controlled, growers should use other good agricultural practices to minimize the risk of contamination *(38)*. The most important vehicle by which people become infected with *S. enterica* is through contaminated food consumption *(23)*. Almost any food can be a vehicle for human salmonellosis infections, and without proper temperature control and sanitation, cross contamination can occur. In studies where proteolyic molds and yeasts were co-inoculated with *Salmonella* species in tomato fruit, increased growth of the mould or yeast increased the *Salmonella* growth *(47, 48)*. When chill injury was imparted on the tomato fruit, *Salmonella* growth increased to higher concentrations then in sound fruit *(47, 48)*. The points of control should focus on contamination prevention and reduction of pathogen survival for each step, up to consumer handling *(14)*.

Conclusions:

The contamination route of *S. enterica* Newport onto tomatoes in the field has not been determined. Several possible routes of contamination have been identified in both the pre and post-harvest environments. Additional research is necessary to determine whether the contamination is due to an environmental condition or occurs because the growers and packers do not follow or have insufficient guidelines to follow GAPs and GMPs. This project hopes to answer questions involving the potential for *S.* Newport to enter the tomato plant and, if so the length of time the bacteria takes to travel from root to fruit. By studying the prevalence of the bacteria in soil samples, farmers will have additional information when preventing produce contamination and additional recommendations can be added to GAPs guidelines for future prevention. Current knowledge of mechanisms of tomato contamination mechanisms and methods of *Salmonella* eradication in produce is inadequate to ensure produce safety. Additional research is necessary to ensure tomato and produce safety from food-borne pathogens.

Table 2.1: Outbreaks associated with *Salmonella* spp. and tomato fruit between 1998 and 2007 in the United States.

Year	Agent	Source	Illnesses (#)
1998	*S.* Baildon	FL	86
2000	*S.* Thompson	FL or GA	29
2002	*S.* Newport	VA	512
2002	*S.* Newport	FL or Mexico	12
2002	*S.* Javiana	FL or Mexico	90
2004	*S.* Javiana	FL, GA or SC	471
2004	*S.* Braenderup	FL	123
2005	*S.* Newport	VA	71
2005	*S.* Enteritidis	CA	77
2005	*S.* Braenderup	FL	76
2006	*S.* Typhimurium	OH	186
2006	*S.* Newport	VA	115
2007	*S.* Newport	VA	65

*Adapted from: Guzewich, J. 2007. Tomato outbreaks 1998-2007. Personal Communication.

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Chapter 3: Association of *Salmonella enterica* serotype Newport with Tomato Plants through Irrigation Water

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ABSTRACT

Tomatoes have been associated with numerous outbreaks of salmonellosis in recent years. Trace back suggests tomatoes may become contaminated during pre-harvest, however exact routes are unclear. The objective of this study was to determine the potential for *Salmonella enterica* serotype Newport to be internalized into the roots, leaves, stems and ultimately fruit of red round tomato plants through contaminated irrigation water, at various stages of plant development, when grown under controlled environmental conditions. Tomato plants were irrigated with 250 or 350 ml (depending on growth stage) of 7 log CFU/ml *S*. Newport contaminated irrigation water every seven days. Presence of the pathogen in plant tissue was evaluated at five growth stages. At each stage, roots, stems, leaves, and two tomato fruit, if present, from four *S*. Newport and four water irrigated (negative control) tomato plants were sampled for *S*. Newport contamination. Association of S. Newport was detected in tomato roots and stems using both conventional plating and molecular techniques. Twenty-four samples were confirmed positive for *S. enterica* using PCR. Sixty-five percent of the roots, 40% of the stems, 5% of the leaves and 5% of the tomatoes sampled were confirmed to contain *S. enterica*. Overall, there was significant difference in the presence of *S.* Newport according to tissue sampled (roots > stems > leaves and fruit) ($P > 0.05$). There was no correlation between growth stage and presence of *S*. Newport in tissues ($P > 0.05$). Ultimately, irrigation with *S*. Newport has a low probability of contaminating tomato fruit.

INTRODUCTION

Fresh produce consumption has increased dramatically over the last two decades. From 1982 to 1997, per capita consumption of fresh produce increased 32% from 91.6 to 121.1 kg per annum *(8)*. The United States is one of the leading producers of tomatoes in the world *(17)*. Approximately 5 billion pounds of tomatoes are consumed annually in the United States *(3)*. Farm value of tomato production is $1.3 billion and the retail value of fresh market tomatoes is estimated to be over $4 billion *(17)*. Since the early 1990s, the annual per capita use of fresh-market tomatoes has dramatically increased because of domestic greenhouses and hydroponic system expansion while the use of processed tomatoes has leveled off *(18)*.

An increase in number of food-borne illnesses linked to fresh produce is coupled with the increase in produce consumption *(8)*. The safety of fresh-market tomatoes and their potential to cause food-borne illness is of growing concern. From 1998 to 2006, 71 produce related outbreaks have been reported, of which 12 were linked to tomatoes *(3, 5)*. Four *Salmonella enterica* serovar Newport outbreaks were linked to tomatoes grown on the Eastern Shore of Virginia *(14)*. *S.* Newport accounts for 42% of *Salmonella* outbreaks in Virginia *(14)*. Without a definitive cause of the outbreaks, the tomato industry seeks possible solutions to prevent and reduce *Salmonella* contamination.

Among the most common reservoirs for *S. enterica* are birds, amphibians, and reptiles. Tomatoes, and other produce, are grown in areas where those animals are found naturally. Sporadic contamination of the tomato fruit could occur through close proximity to these natural reservoirs.

Microorganisms can enter tomato plants through various pathways. Potential *Salmonella* entry routes for tomato plants could include roots, flowers, or into the fruit via the stem scar or cracks in the cuticle. Conflicting research exists regarding whether entry into the plant tissues results in contaminated fruit. When Jablasone *et al.* applied contaminated water directly onto the soil of pots containing cultivar Cherry Gold tomatoes, *S.* Enteritidis was not recovered from the stems, leaves, or fruits of tomato plants *(15)*. Additional *S.* Montevideo irrigation studies performed on greenhouse tomatoes, found no

contamination in tomato fruit, but samples of roots exposed to 4 or more irrigation events (one every 14 days) were positive *(19)*.

Inoculation of *Salmonella* into aerial plant tissues resulted in contaminated tomato fruits. Guo *et al.* showed that a cocktail of five *S. enterica* serotypes (Montevideo, Michigan, Poona, Harford, and Enteriditis), either inoculated onto tomato flowers or injected into the stem, caused 37% of fruit to test positive for *Salmonella (11)*. *S. enterica* is capable of survival on and in tomato fruit *(16)*. Lin *et al.* found that *S.* Montevideo is able to survive on tomato stems and cracks in tomato fruit *(16)*.

Tomato fruit could also become contaminated during post-harvest processing. After harvest, tomato fruits are commonly deposited in a water filled dump tank and transported through a flume system. Both the dump tank and flume water are typically treated with chlorine, however the chlorination treatments are completed primarily to maintain water quality *(23)*. Treatment of tomato fruit with chlorinated water cannot completely eliminate microorganisms within the interior of the fruit, thus making it difficult for this treatment to ensure tomato safety *(23)*. Currently, no effective post-harvest kill step exists for removing *S. enterica* from tomato fruits. Thus, prevention of *S. enterica* introduction into tomato plants is essential for preventing future outbreaks.

In 2007, the United States Food and Drug Administration (FDA) implemented a multiyear initiative aimed at reducing tomato related illnesses *(9)*. Goals of this initiative include identifying production practices or conditions, which may lead to tomato contamination, as well as facilitating and promoting tomato safety research *(9)*. This study hopes to mimic natural growing conditions as closely as possible while continuing the FDA's efforts by determining whether tomato plants irrigated with *S.* Newport have the potential to uptake the organism into roots, stems, leaves, or fruit of the plant resulting in contaminated tomato fruit. The increasing number of outbreaks has raised concerns about the pre-harvest *S. enterica* colonization of tomatoes *(9)*. Understanding whether or not this phenomenon occurs may provide more complete understanding of *Salmonella* entry points and help determine mitigation strategies, which could prevent tomato related salmonellosis outbreaks in the future.

Materials and Methods

Bacterial Culture:

A clinical isolate of *Salmonella enterica* serotype Newport J1893, isolated from a human Salmonellosis case associated with the 2005 tomato related outbreak, was obtained from the Centers for Disease Control and Prevention (CDC) (Atlanta, GA) and utilized in this study.

The frozen isolate was plated for isolation onto XLT-4 agar (Becton Dickson, Sparks, MD) and incubated for 24 hours at 37°C, for preliminary confirmation of *Salmonella* species. Colonies presumptively positive for *Salmonella* spp. were red with black centers indicating H_2S production. The isolates' serotype was confirmed using the slide agglutination test (Becton Dickson, Sparks, MD). A positive result was indicated when clumping occurred between the antiserum and the suspension. Agglutination with antiserum group C2 was confirmed as *S.* Newport. The confirmed strain was grown in Tryptic Soy broth (TSB) (Difco, Detroit, MI) then stored in 30% glycerol and TSB solution at -80 ºC in an ultra low freezer in the Food Science and Technology Department.

Inoculum preparation:

To begin each experiment, cells were activated by three successive 24 hour incubations at 37ºC in TSB. The final suspension was centrifuged at 7,500 X g for 10 minutes and the spent medium decanted. The culture was re-suspended in sterile de-ionized (DI) water to create a final 7 log CFU/ml *S.* Newport suspension. Each 250 ml bottle was taken from a batch of 1800 ml 7 log CFU/ml prepared suspension to individually water each plant.

The volume of water used to irrigate the tomato plants was dependent on the soil capacity and growth stage of the tomato plants. Initially, transplants were irrigated with 250 ml 7 log CFU/ml *S.* Newport suspension. As the plants began to flower, they received 350 ml 7 log CFU/ml *S*. Newport suspension, to account for the increased plant size and additional water requirements. Each batch of the *S.* Newport suspension water was enumerated onto XLT-4 agar immediately before watering tomato plants to confirm inoculum concentration using surface plating, with 0.1 ml decimal dilution. Plates were

incubated at 37°C for 24 hours.

Tomato Growth and Irrigation Parameters:

Commercially produced seven week old tomato seedlings (Solar Fire cultivar) were obtained from Virginia Tech's Eastern Shore Agricultural Research and Extension Center (ESAREC, Painter, VA) in a styrofoam plug tray with Miracle Grow®: Seed Starting Potting Mix (Marysville, OH). Forty-four seedlings were planted in steam sterilized sandy loam BOJAC series soil obtained from ESAREC in 15.6 L (3 gallon) sterilized round terra cotta pots and evenly distributed between two growth chambers. The soil was kept moist but not saturated, to prevent the soil from becoming an anaerobic environment. Each pot was individually placed in a polypropylene tray to collect the irrigation runoff.

Tomato plants were grown in a Conviron CMP5090 (Winnipeg, Canada) reach-in controlled environmental growth chamber (8'x 3'x 8') under a day and night cycle of 12 hours. The daytime temperature was maintained at 28°C, with relative humidity of 60%, and the nighttime temperature of 26°C. Plants were divided into two treatment groups: control (irrigated with distilled water) and contaminated water inoculated (irrigated with *S.* Newport). All plants regardless of treatment group received daily watering with 250 ml distilled water until flowering stage was reached. Each tomato plant received an irrigation treatment applied individually to the top soil. After flowering stage, tomato plants received 350 ml distilled daily watering treatment. Once every seven days, the tomatoes from the contaminated water experimental group were watered with 7 log CFU/ ml *S.* Newport inoculum instead of distilled water. Two growth chambers were grown consecutively. One growth chamber contained 22 control tomato plants and a second growth chamber contained 22 contaminated water inoculated tomato plants. Both CMP5090 growth chambers were held to the same relative conditions. Stakes were inserted into the pots and string secured the stems, to ensure vertical growth. Tomatoes received a Miracle Grow®: Tomato Plant Fertilizer (Marysville, OH) treatment, per manufacturer's instructions, once a week, or as chlorosis developed, throughout the growing period.

Tomato Plant Sampling:

The tomato plants were randomly sampled at various tomato growth stages 14 days

post transplant, early fruit (49 days post transplant), mid fruit (56 days), full fruit (65 days), and terminal (67 days) to track the association of *S.* Newport through the plant over time. The growth stage 14 days post transplant sampling occurred 14 days after the seedlings were transplanted into the BOJAC soil. Early fruit were sampled when the plants had two or more green fruit. Sampling of mid fruit occurred when the plants contained a variety of red and green fruit. Full fruit sampling occurred as the plants contained all red fruit and terminal stage was sampled as the plant appeared to wilt and the tomato plant began terminating growth. At each sampling time, four randomly selected plants from each treatment group (8 plants total) were dissected and the roots, stems, leaves, and fruit (if present) were sampled for the presence of *S*. Newport. Conventional plating and molecular procedures were used to analyze the plant tissues for *S.* Newport.

Stem and Leaf Analysis:

Randomly selected leaves were aseptically removed from the plant were selectively removed using surface sterilized scissors. Leaves from one branch from the top, middle, and lower portions of the tomato plant were included. The three branches were surface sterilized by spraying with 70% ethanol solution and allowed to dry under a flow hood until no visible solution remained *(19)*. The leaves were aseptically combined in one stomacher bag and treated as a single sample. The stems were aseptically cut in 3 cm portions beginning at 20, 40, and 60 cm and combined to form one sample *(19)*. If the stem was not long enough to sample in intervals, the entire stem was sampled. The stem was surface sterilized by spraying with 70% ethanol solution and allowed to dry under a flow hood until no visible ethanol remained *(19)*. Each sample was combined with 10 ml of 0.1% buffered peptone water and stomached for 90 seconds. Both the stem and leaf homogenates were streaked (10 µl) onto XLT-4 agar. The plates were incubated at 37 ºC for 24 hours. Potential positive *S*. Newport colonies were observed and recorded.

Root Analysis:

Roots were washed in distilled water, until no residual soil remained. The roots were surface sterilized by spraying with 70% ethanol solution and allowed to dry under a laminar flow hood until no visible ethanol remained *(19)*. The root sample was then

combined with 10 ml of 0.1% buffered peptone water and stomached for 90 seconds. The homogenate was streaked onto XLT-4 agar. The plates were incubated at 37 ºC for 24 hours. Potential positive *S*. Newport colonies were observed and recorded.

Tomato Analysis:

Both green and red ripe tomatoes were harvested from plants. Tomatoes were surface sanitized by spraying 70% ethanol solution and then allowed to dry under a laminar flow hood, until no visible ethanol remained *(19)*. The tomatoes were then placed in individual sterile stomacher bags containing 10 ml 0.1% peptone water and stomached for 90 seconds. The homogenate was streaked onto XLT-4 agar. The plates were incubated at 37 ºC for 24 hours. Presumptive positive *S*. Newport colonies were observed and recorded.

Enrichment procedures:

The homogenates from all root, stem, leaf, and fruit samples were enriched for *S*. Newport. One ml aliquots of the homogenates were separately inoculated into 10 ml Tetrathionate broth (Remel, Lenexa, KS). Tetrathionate broth was incubated at 37°C for 24 hours then streaked onto XLT-4 agar. After 24hour incubation at 37°C, typical *S*. Newport colony formation identified presumptive positives.

Polymerase Chain Reaction Protocol for Confirmation of *S. enterica* in Samples:

Following enrichment procedures, presumptive positive colonies were confirmed using polymerase chain reaction procedures (PCR). Each presumptive colony was grown to 7 log CFU/ml in 10 ml TSB at 37°C for 24 hours, prior to PCR analysis. The culture was centrifuged at 7,500 x g for 10 minutes then re-suspended in sterile DI water. DNA was extracted using the Qiagen Generation Capture Plant Kit (Qiagen, Valencia, CA), per manufacturers instructions. The Nanodrop 1000 (NanoDrop Industries Inc., Wilmington, DE) confirmed the presence and concentration of DNA obtained from each sample following extraction. A "master mix" of PCR reagents was prepared containing 0.05 µM *invA* (invA-112F 5'- TCGACAGACGTAAGG 3' and invA-1920R 5'- GCGATATTGGTG-3', 100ng of purified DNA, and 2x Taq Mastermix (New England Biolabs, Ipswich) (3). PCR conditions were as follows: initial denaturation at 96 °C for

two minutes, followed by 35 cycles of: 96°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute and a final extension of 10 minutes at 72°C (3). The potential positive isolates that were negative for *invA* were further analyzed using 16S rDNA in order to confirm that there was DNA in the extraction sample and that the DNA was amplifiable.

Gel Electrophoresis:

To visualize the DNA fragments, gel electrophoresis was used to separate PCR fragments by size on a 1.5% agarose gel, in 1x tris-acetate EDTA (TAE) buffer (Fisher BioReagents, Pittsburgh, PA), and a 0.5 µg/ml concentration of ethidium bromide (Fisher BioReagents, Pittsburgh, PA). Each 10µl sample was loaded into a well on the gel with Blue/ Orange 6X loading dye (Promega, Madison, WI). A 100 bp DNA ladder (Promega, Madison, WI) served as a marker. The gel was visualized using UV light and a band size of 1800 confirmed amplification of the invA gene and this isolation of *S. enterica*.

Statistical analysis:

Each treatment group contained 22 tomato plants (total of 44 plants) and four plants from each group were sampled at random at each of the five growth stages. Two tomato fruit from each plant were sampled separately. The correlation between the plant tissue type (root, stem, leaf or fruit) and growth stage (14 days, early fruit, mid fruit, full fruit or terminal) on the presence of *S. enterica* was evaluated using Chi-Square analysis. Statistical analysis was completed using Statistical Analysis Software version 9.1 (SAS Institute, Cary, NC).

Results and Discussions:

The presence of *Salmonella enterica* serotype Newport was detected in both the tomato roots and stems but was not consistently detected in the tomato leaves or fruit (Table 3.1, Table 3.2). Of the plants irrigated with *S*. Newport, 32% (29 out of 92) of samples before enrichment and 39% (36 out of 92) after enrichment were presumptively positive for *S*. Newport (Table 3.1, Table 3.2). Of the 29 presumptively positive pre-enrichment samples, 69% were root samples, 24% were stem samples, and 7% were leaf samples (Table 3.1). Following enrichment procedures, an additional five stem and two

tomato fruit samples were presumptive *S.* Newport positive after enrichment (Table 3.2). All plants irrigated with distilled water exclusively (control) were *S*. Newport negative (Table 3.1 and Table 3.2).

In order to confirm that isolates were *S. enterica*, all enriched homogenates amplified for the *invA* gene (exclusive to *S. enterica*) using PCR (Table 3.3, Fig. 3.1, Fig, 3.2, Fig. 3.3). Previous work in the Ponder lab established at the *invA* primers are specific for only members of *S. enterica*, and do not amplify other members of the *Enterobactereace*. Following PCR, 13 of 20 of presumptive positive roots samples, 8 of 12 stem samples, 1 of 2 leaf samples and 2 of 2 fruit samples were confirmed for *S. enterica* (Table 3.3, Fig. 3.1, Fig, 3.2, Fig. 3.3). Therefore, 65% of roots, 40% of stems, 5% of leaves and 5% of fruit were contaminated following irrigation with *S*. Newport (Table 3.3, Fig. 3.1, Fig, 3.2, Fig. 3.3). PCR was able to confirm *S. enterica* in all but 12 presumptive positive samples (Table 3.3, Fig. 3.1, Fig, 3.2, Fig. 3.3). The homogenate samples, which were negative for *S. enterica* following PCR were further analyzed using the 16S rDNA to ensure that DNA was amplified in those samples. Overall, significantly more samples of roots were positive for *S. enterica* than the stems, and significantly more stems were positive than the leaf and fruit samples taken ($P \leq 0.05$). There were no significant differences ($P \geq 0.05$) between the growth stage of the plant and presence of *S. enterica* in any of the tissues.

This study showed *S*. Newport may associate with the roots of tomato plants through contaminated irrigation water. All samples were surface sanitized in hopes of only detecting internalized microorganisms. All 20 contaminated irrigation root samples were potentially *S*. Newport positive using standard plating methods (Table 3.1 and Table 3.2). Hydroponically grown tomato plants have shown *Salmonella* root internalization *(13)*. Within one day exposure of plant roots to a nutrient solution, hypocotyls, cotyledons, and stems, for plants with intact root systems, showed positive *Salmonella* internalization *(13)*. When portions of the roots were removed, hypocotyls and cotyledons again showed positive internalization *(13)*. Regardless of root condition, hypocotyls, cotyledons, stems, and leaves of plants grown for 9 days showed positive *Salmonella* internalization *(13)*. Miles et al. irrigated six tomato treatment groups with *S.* Montevideo every 14 days for 70 days, with each group receiving an increased number of contaminated watering events

(19). The tomatoes were not sampled during growth, only at terminal fruiting. *S.* Montevideo was unable to internalize in stems, leaves, or tomato fruit after contaminating irrigation water, however the roots from plants that had 4, 5 and 6 contaminated irrigation water events, were positive for *S. enterica (19)*. However, additional work completed by Jablesone *et al.* found no significant evidence of *Salmonella* internalization in stem, leaves, and tomato fruit samples after irrigating the tomato plants with contaminated water *(15)*.

S. Newport can internalize into other produce varieties roots and leaves. When romaine lettuce was grown in contaminated potting medium, roots and leaves were positive for *S*. Newport *(2)*. Restriction of bacterial root uptake then later transmission to the above ground parts of the plant might have been due to impermeability of the root surface to bacteria, restricted loading into the xylem, or immobility throughout the plant *(2)*. Lettuce plants were sampled after root tips were removed to expose the vascular cylinder and possibly allow *Salmonella* to penetrate and translocate through the lettuce plants *(2)*. Internalization occurred regardless of whether roots were damaged or intact, however greater *S*. Newport numbers were shown when the roots were mechanically damaged *(2)*.

Plant developmental stage can also effect plant tissue internalization of bacteria. *S*. Newport was detected in 33 day old lettuce plants, but not 17 days or 20 days plants, suggesting the plants developmental stage effects bacterial internalization *(2)*. *S. enterica* was also found in sterilized leaf samples from lettuce plants grown in contaminated soil *(10)*. Researchers hypothesize that exudation of nutrients at root junctions could attract human pathogens to these sites for plant internalization but could depend on plant type *(4, 6)*. Very little information is known concerning the factors involved in plant-pathogen interactions and how the human pathogen can uptake into the roots then translocate throughout the plant *(2)*.

Previous tomato studies have shown *Salmonella* internalization into the stems, leaves, and tomato fruit after structural damage or direct inoculation into the stem or flowers occurred *(12, 15)*, however this study demonstrated internalization into the stem occurred in 40% (8/ 20) of samples (Table 3.1 and Table 3.2).

In this study, *S*. Newport was less likely to be associated with leaves and fruit of tomatoes. Aerial plant tissue, such as leaves, have a greater degree of initial contamination

but once contaminated, the pathogens encounters harsh physiochemical conditions that diminish their survival rate *(7)*. *S. enterica* numbers on leaf tissue was 30 to 40 fold less than those detected on roots *(4)*. If accidental *S*. Newport aerosolization did occur during the experiment, the bacteria could have located on the leaf base at the trichomes, outer rim of stomates, or the cell grooves along the veins, where an increased survival rate exists *(20)*. At these sites, bacterial communities resembling biofilms flourish compared to the un-colonized part of a leaf *(20, 21)*. Under conditions where the root system is not disturbed, *S*. Newport contaminated irrigation water applied to the soil has a low probability of contaminating tomato fruit *(1, 15)*. The experiment was conducted under simplistic conditions not representative of the complex natural systems found in agricultural fields *(7)*.

Competition between human pathogens and indigenous microbial flora affect internalization of pathogens *(22)*. *Enterobacter cloacae* was detected in two of three seedlings before transplant occurred, this was confirmed using a 20E API strip (bioMerieux, Marcy l'Etoile, France). The same microorganism was detected throughout the experiment on XLT-4 agar and used as a confirmed negative control during molecular techniques. *E. cloacae* possibly contributed to the increased S. Newport tomato plant association *(12)*. The *E. cloacae* association was not quantified but research suggests a direct relationship between indigenous microbial flora and human pathogen numbers *(6)*.

Overall, *S*. Newport may be capable of contaminating tomato plants throughout their growth stages. *S*. Newport was able to contaminate the plant structures at every growth stage, demonstrating the possibility for irrigation water to affect the long term contamination of tomato plants.

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Tables and Figures:

Table 3.1: Number of tomato plant samples (roots [R], stems [S], leaves [L], and fruit [F1, F2]) presumptively positive for *S. enterica* serotype Newport (prior to enrichment of homogenate) in plants irrigated with water (control) and *S.* Newport at different growth cycle stages, grown under controlled environmental conditions.

	Irrigated with Water (n=4)					**Irrigated with *S.* Newport (n=4)**				
	R	**S**	**L**	**F1**	**F2**	**R**	**S**	**L**	**F1**	**F2**
14 Days	0	0	0	N/A	N/A	4	0	1	N/A	N/A
Early Fruit	0	0	0	0	0	4	2	0	0	0
Mid Fruit	0	0	0	0	0	4	3	1	0	0
Full Fruit	0	0	0	0	0	4	1	0	0	0
Terminal	0	0	0	0	0	4	1	0	0	0

*14 days post transplant sampling occurred 14 days after the seedlings were transplanted into the BOJAC soil. Early fruit were sampled when the plants had two or more green fruit (49 days). Mid fruit were sampled when the plants contained a variety of red and green fruit (56 days). Full fruit sampling occurred as the plants contained all red fruit (65 days) and terminal stage (67 days) was sampled as the plant appeared when wilting and began terminating growth.

Table 3.2: Number of tomato plant samples (roots [R], stems [S], leaves [L], and fruit [F1, F2]) presumptively positive for *S. enterica* serotype Newport (after enrichment) in plants irrigated with water (control) and *S.* Newport at different growth cycle stages, when the plants were grown under controlled environmental conditions.

	Irrigated with Water (n=4)					**Irrigated with *S.* Newport (n=4)**				
	R	**S**	**L**	**F1**	**F2**	**R**	**S**	**L**	**F1**	**F2**
14 Days	0	0	0	N/A	N/A	4	1	0	N/A	N/A
Early Fruit	0	0	0	0	0	4	4	1	0	0
Mid Fruit	0	0	0	0	0	4	3	1	1	0
Full Fruit	0	0	0	0	0	4	3	0	1	0
Terminal	0	0	0	0	0	4	1	0	0	0

*14 days post transplant sampling occurred 14 days after the seedlings were transplanted into the BOJAC soil. Early fruit were sampled when the plants had two or more green fruit (49 days). Mid fruit were sampled when the plants contained a variety of red and green fruit (56 days). Full fruit sampling occurred as the plants contained all red fruit (65 days) and terminal stage (67 days) was sampled as the plant appeared when wilting and began terminating growth.

Table 3.3: Number of tomato plant samples (roots [R], stems [S], leaves [L], and fruit [F1, F2]) confirmed positive for *S. enterica*, using molecular techniques, in plants irrigated with water (control) and *S.* Newport at different growth cycle stages, grown under controlled environmental conditions.

	Irrigated with *S.* Newport (n=4)				
	R	**S**	**L**	**F1**	**F2**
14 Days	2	2	0	N/A	N/A
Early Fruit	2	4	0	0	0
Mid Fruit	2	1	1	1	0
Full Fruit	4	0	1	1	0
Terminal	3	1	0	0	0

*14 days post transplant sampling occurred 14 days after the seedlings were transplanted into the BOJAC soil. Early fruit were sampled when the plants had two or more green fruit (49 days). Mid fruit were sampled when the plants contained a variety of red and green fruit (56 days). Full fruit sampling occurred as the plants contained all red fruit (65 days) and terminal stage (67 days) was sampled as the plant appeared when wilting and began terminating growth.

Figure 3.1: PCR results (*invA* primers) confirming presumptive positive *S. enterica* isolated from root (Lane 1, Lane 2, Lane 3, Lane 4, Lane 7, Lane 8, Lane 9, Lane 10), stem (Lane 5, Lane 6, Lane 11, Lane 12, Lane 13, Lane 14), and leaf (Lane 15). Gel includes positive *S. enterica* control (Lane 17), and Promega 100 bp genomic ladder.

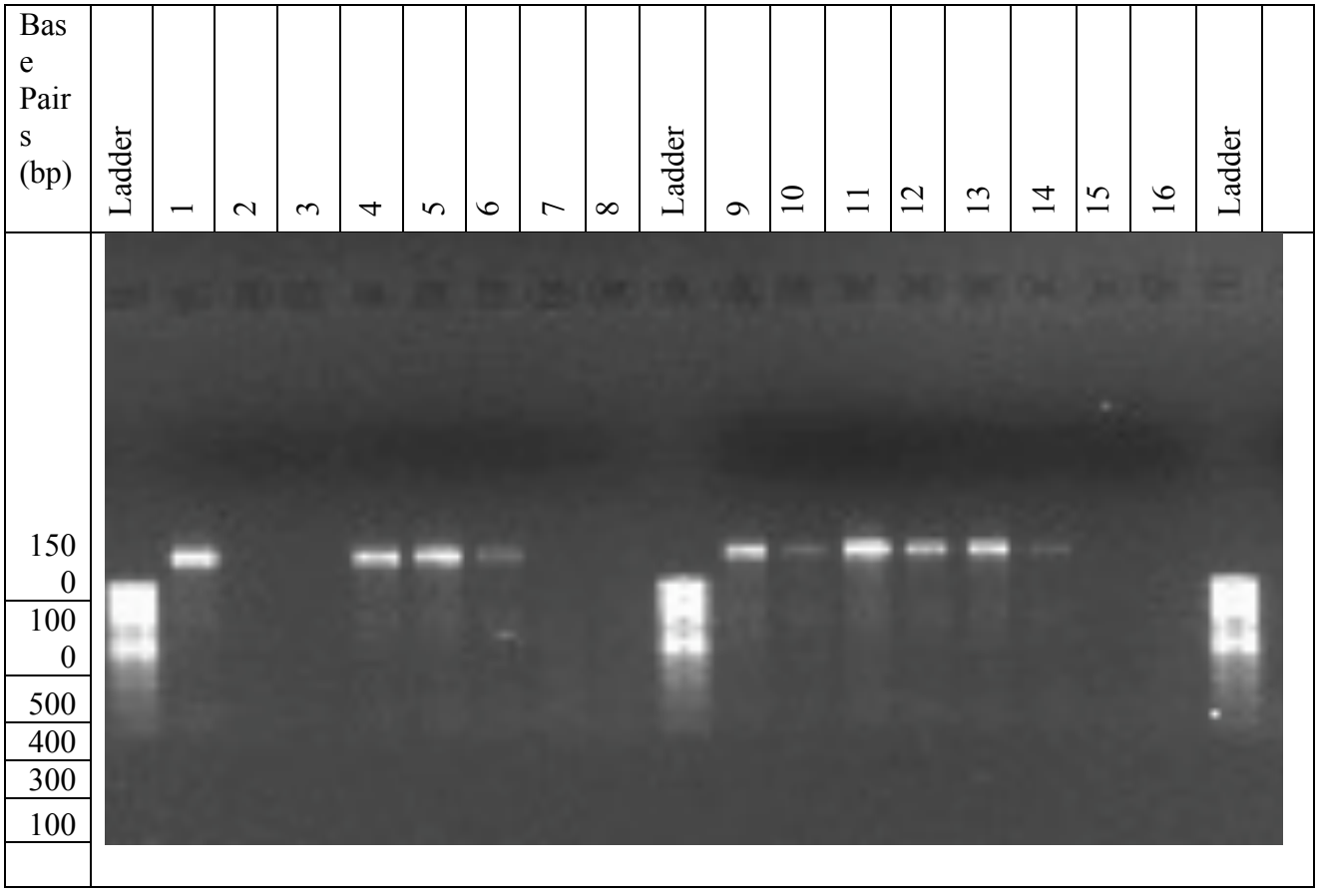


Figure 3.2: PCR results (invA primers) confirming presumptive positive S. enterica isolates from root (Lane 17, Lane 19, Lane 20, Lane 21, Lane 27, Lane 28, Lane 29, Lane 30), stem (Lane 22, Lane 23, Lane 24, Lane 31), leaf (Lane 25), and fruit (Lane 26) samples Gel includes Negative control (Lane 18), and Promega 100 bp genomic ladder.

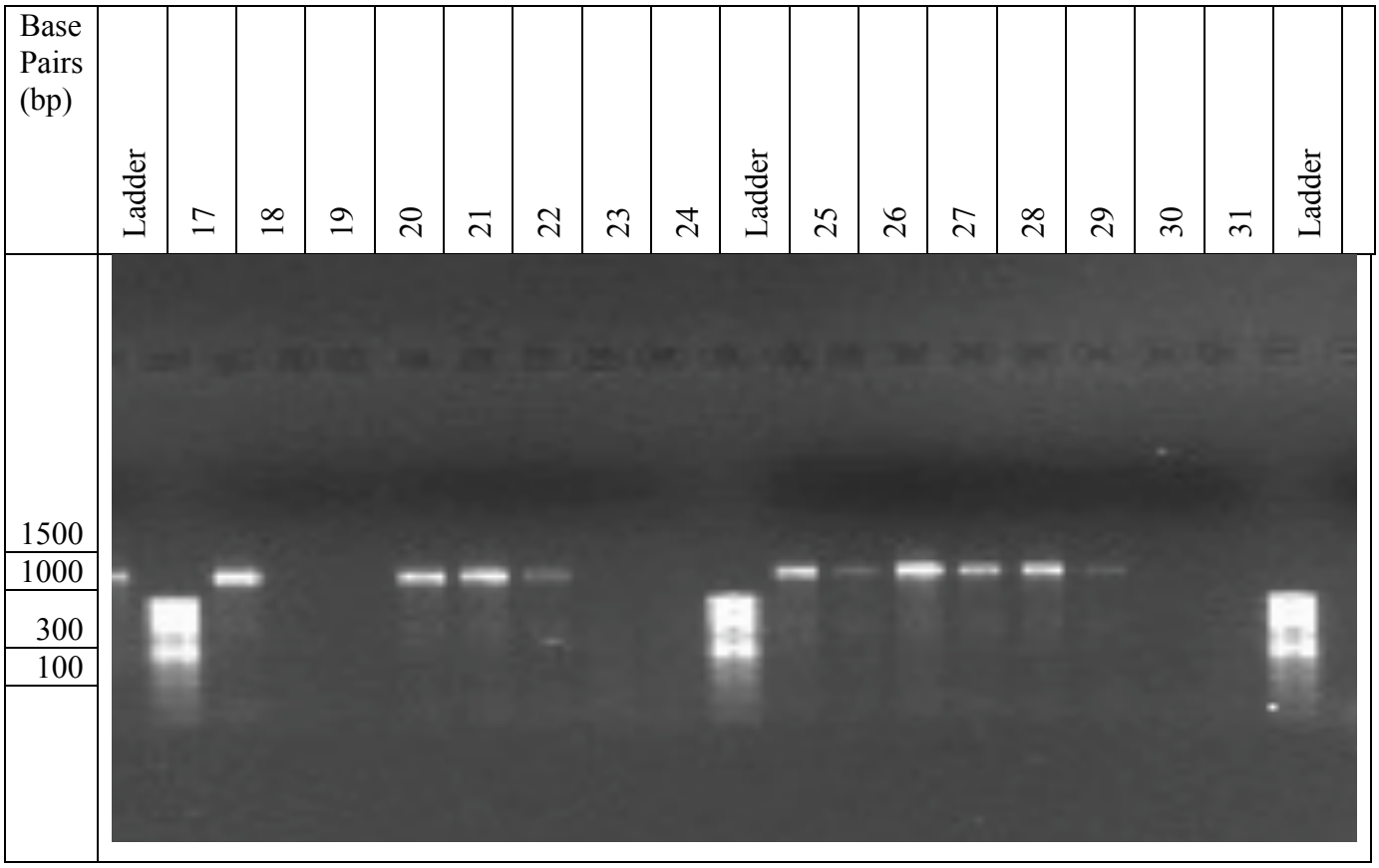
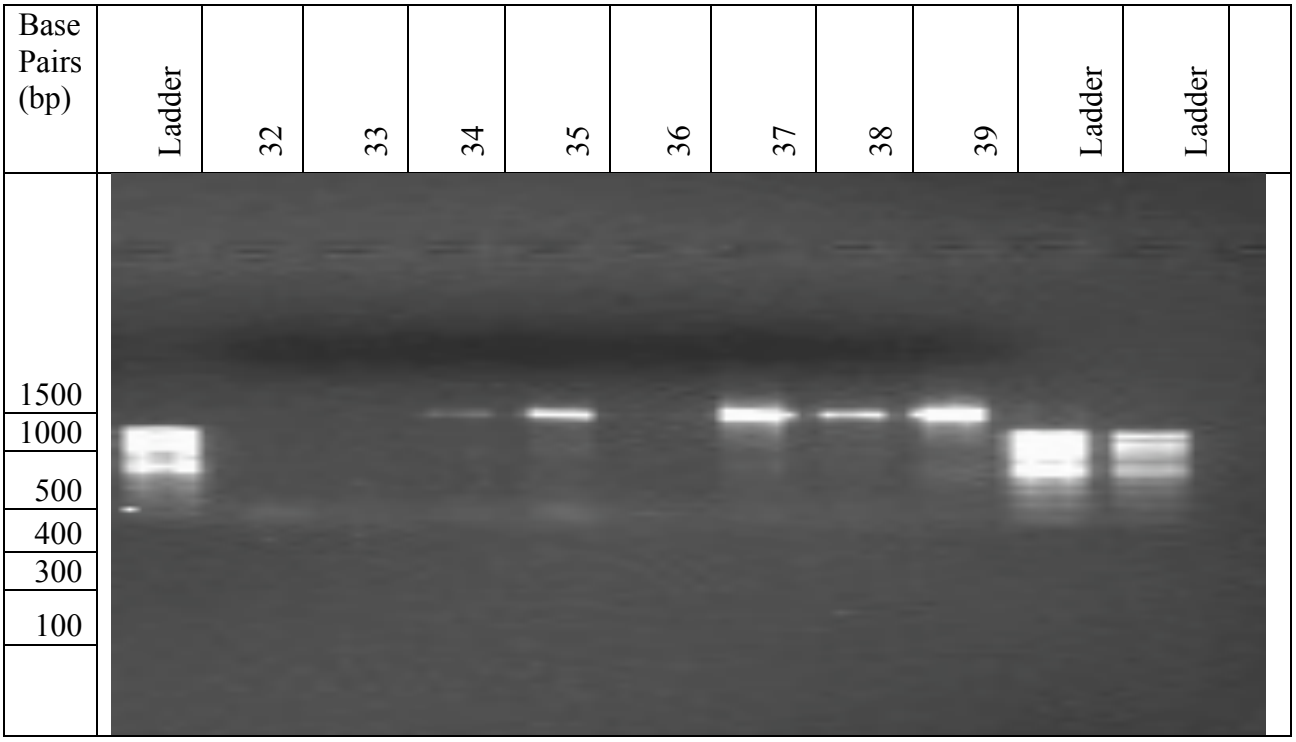


Figure 3.3: PCR results (invA primers) confirming presumptive positive *S. enterica* isolates from root (Lane 35, Lane 36, Lane 37, Lane 38) stem (Lane 32, Lane 33, Lane 39), and fruit (Lane 34). Gel includes Promega 100 bp genomic ladder.



Chapter 4: Conclusions

This work addresses issues involving the safety of fresh produce contaminated through irrigation water, grown under controlled environmental conditions. Specifically, evaluating the potential for high levels (7 log CFU/ml) of *Salmonella enterica* serotype Newport to be internalized into tomato plant tissues via contaminated irrigation water at different developmental growth stages of the plant. For this experiment, tomatoes were grown in an environmentally controlled growth chamber since agricultural field studies with *S*. Newport is not possible. Overall, *S*. Newport may be capable of contaminating tomato plants throughout their growth stages. The presence of *S. enterica* was confirmed in 70% of root samples, and40% stem samples but was not consistently detected in the tomato leaves (1 of 20) or fruit (2/40). *S*. Newport was able to contaminate the plant structures at every growth stage, demonstrating the possibility for irrigation water to affect the long-term contamination of tomato plants.

Limitations and Pitfalls:

The experiment was conducted in two separate growth chambers to minimize cross contamination between experimental groups without focusing on statistical significance. In previous research (Appendix A), when *S*. Newport irrigated and control plants were grown together, control plants continued to be contaminate. Great care was taken to prevent this phenomena, however it was unable to be controlled. *S*. The mechanism for contamination of the control plants in our previous experiments is unknown. This experiment isolated tomato plants in two separate growth chambers by treatment group in order to minimize between group interactions. While this successfully prevented control contamination, results between each treatment group stand alone. When each growth stage was reached, both treatment groups were sampled at the same time. Slight discrepancies might exist between chambers and account for slight growth fluctuations.

Discrepancies exist between the association shown in this experiment and the Appendix, including association seen in negative control tomato plants and the degree of association throughout the growing phases. A cause was not confirmed but could be speculated that the soil or microbiological aerosolization could affect the *S*. Newport association. In the Appendix, BOJAC series soil was obtained from the AREC center in

Painter, VA and the soil was used with no treatment. During this experiment, soil from the same location received a steam sterilization treatment. It is unknown whether the soil treatment had an effect on *S*. Newport association. Despite careful efforts, human error might have accidentally spread the contamination to control plants or aerial plant parts, while irrigating with *S*. Newport. Native or dormant soil microorganisms, that escaped the steam sterilization treatment, could contribute to *S*. Newport association but the effect is unknown.

Future Research:

This experiment investigates the relationship between *S*. Newport contaminated irrigation water and the subsequent pathogen association with a tomato plant. As discussed in the limitations, accidental *S*. Newport aerosolization might have increased the spread of contamination. To ensure this was not the case, future research should devise methodology to minimize aerosolization, through soil covers or dividers between plants and treatment groups, within the growth chamber. To determine the relationship between sterilized soil, non-sterilized soil, and association researchers should investigate whether native soil microorganisms affect *S*. Newport and other human food-borne pathogens association with tomato plant structures. Alternatively, researchers should investigate whether mechanical root damage, time between contamination irrigation treatment and sampling, or native bacteria, such as *E. cloacae*, contribute to *S*. Newport association and at what growth stage these factors have the most detrimental effect. The tomato plants were grown under control environmental conditions and future research should use an indicator microorganism in an agricultural field setting, to determine large-scale reproducibility.

Appendix A: Preliminary data and trouble shooting procedures for study: Association of *Salmonella enterica* serotype Newport with Tomatoes Grown Under Controlled Environmental Conditions

Materials and Methods:

Bacterial Culture:

Clinical isolates of *Salmonella enterica* serotype Newport, from tomato related outbreaks, obtained from the CDC, were utilized in this study. Five *S.* Newport isolates, J1890, J1891, J1892, J1893 and J1894, were compared using a growth curve and the most viable strain was selected for the study. The isolates were stored at -80°C in an ultra low freezer in the Food Science & Technology Department.

Overnight cultures of the *S.* Newport isolates were transferred three times in Tryptic Soy broth (TSB) (Difco, Detroit, MI) to separately activate the cells. After the last transfer, the strains was plated onto Hektoen Enteric agar (HEA) (Difco, Detroit, MI) and incubated for 24 hours at 37°C, for preliminary confirmation of *Salmonella* species. Colonies positive for *Salmonella* spp. were blue green to blue with black centers indicating H_2S production. The five isolates were confirmed for serotype using the slide agglutination test (Becton Dickson, Sparks, MD). Two separate one inch square sections were drawn onto a slide with a wax pencil. A drop of saline was placed in each square section and a single representative colony from HEA was emulsified. In one square section, a drop of *Salmonella* O Antiserum group C2 was added. To the other section a drop of saline was added to serve as a negative control. The glass slide was gently rocked for one minute. A positive result was indicated when clumping occurred between the antiserum and the suspension. Agglutination with antiserum group C2 was confirmed membership to the same O group as *S.* Newport.

Growth curve studies were performed to determine the most viable *S.* Newport isolate. 100 ml TSB was inoculated with a loopful (100 µl) of a 24 hour confirmed *S.* Newport strain TSB suspension. Absorbance readings were taken every 30 minutes from a Shimadzu UV-2101PC spectrophotometer (Columbia, MD) using a 1cm pathlength cuvette (Plastibrand, Werthelm, Germany). Readings were taken until the culture reached exponential growth, then readings were taken every hour until the culture reached high

exponential growth. The procedure was repeated for each confirmed *S.* Newport strain. A growth curve was constructed to compare the optical densities of each strain over time. The slope of each exponential growth phase was calculated and the most viable strain was selected based on the largest slope. The strain selected was used throughout the remaining experiments.

The most viable *S.* Newport strain, J1893, was made nalidixic acid resistant by plating onto Tryptic Soy agar (TSA) (Difco, Detroit, MI) with increasing concentrations of nalidixic acid (5, 10, 25, 50ppm) (Agros Organics, New Jersey) and transferring isolated colonies every 24 hours. Repeated transfers occurred until colonies were nalidixic acid resistant at a level of 50ppm. The strain was then streaked onto TSA supplemented with 50ppm nalidixic acid (TSAN) for confirmation.

Inoculum preparation:

To begin each experiment, *S.* Newport isolate J1893 was transferred into TSB and activated as described previously. The final suspension was grown in TSB for 24 hours at 37°C then centrifuged at 7,500 X g for 10 minutes and the spent medium decanted. The culture was re-suspended in sterile de-ionized (DI) water to create a final 7log CFU/ml *S.* Newport suspension.

The amount of water used to irrigate the tomato plants was dependent on the soil fluidity and growth stage of the tomato plants. Preliminarily, the *S.* Newport irrigated tomato plants were watered with 250 ml 7 log CFU/ml *S.* Newport suspension. Each 250 ml bottle was taken from a batch of 1800 ml 7 log CFU/ml prepared suspension to individually water each plant. As the plants began to flower, the tomato plants received 350 ml, to account for the increased plant size and additional water requirements, of a 7 log CFU/ml *S.* Newport suspension. Each batch of the *S.* Newport suspension water was enumerated onto HEA for trial 1, or XLT-4 (Becton Dickson and Company, Sparks, MD) for trail 2, immediately before watering the tomato plants. Plates were incubated at 37°C for 24 hours.

Tomato Growth and Irrigation Parameters:

Applause hybrid red round tomato seeds were used for this study. Applause seeds were planted in a 50count plug tray with Miracle Grow®: Seed Starting Potting Mix (Marysville, OH). Once the seeds reached a three true leaf stage, approximately 10 days, 22 tomato seedlings were selected for transplant based on color, health, and overall appearance. The 22 seedlings were planted in sandy loam BOJAC series soil obtained from the Eastern Shore Agricultural Research Center (Painter, Virginia) in 15.6 L (3 gallon) plastic pots and placed in a growth chamber. Preliminary BOJAC soil analysis was done at the Virginia Tech Soil Testing Lab (Table 1) to analyze soluble salts, organic matter, and other routine tests (pH, P, K, Ca, Mg, Zn, Mn, Cu, Fe, B, and estimated CEC). The soil was kept moist but not saturated, to prevent the soil from becoming an anaerobic environment. Each pot was individually placed in a polypropylene tray to collect the irrigation runoff.

Tomatoes were grown in a Conviron CMP5090 (Winnipeg, Canada) reach-in controlled environmental growth chamber (8'x 3'x 8'), under a day and night cycle of 12 hours. The daytime temperature was 28°C, with a constant relative humidity of 60%, and the nighttime temperature of 26°C. Plants were divided into two treatment groups: control (irrigated with distilled water) and contaminated water inoculated (irrigated with *S.* Newport). All plants regardless of treatment group received daily watering with 250 ml distilled water until flowering stage was reached. Tomato plants then received a 350 ml distilled daily watering treatment. Once every seven days, the tomatoes from the experimental group were watered with 7log CFU/ ml *S.* Newport inoculum instead of distilled water. Two growth chambers were grown consecutively. Growth Chamber 1 began on October 20, 2007 and terminated March 3, 2008. Seeds were planted in Growth Chamber 2 on November 16, 2007 and ended March 8, 2008. The seeds for Growth Chambers 3 and 4 began on April 8, 2008 and terminated June 16, 2008. Stakes were inserted into the pots and string secured the stems, to ensure vertical growth. Tomatoes received a Miracle Grow®: Tomato Plant Fertilizer (Marysville, OH) treatment once a week, or as yellow coloring developed, throughout the growing period.

Tomato Plant Sampling:

The tomato plants were randomly sampled at various tomato growth stages (14 days

Post Transplant, Early Fruit, Mid Fruit, Full Fruit, and Terminal) to track the association of *S.* Newport through the plant over time. At each sampling time, two randomly selected plants from each treatment group (4 plants total) were dissected and samples taken from the roots, stems, leaves, and fruit, if present. Conventional plating and molecular procedures were used to analyze the plant tissues for *S.* Newport.

Stem and Leaf Analysis:

The leaves were aseptically removed from the plant by randomization. Randomization selected one branch from the top, middle, and lower portions of the tomato plant. The stem and leaves were surface sterilized by spraying with 70% ethanol solution and allowed to dry under a flow hood until no visible ethanol remains. The stems were sampled in 3 cm portions beginning at 20, 40, and 60 cm. If the stem is not long enough to sample in intervals, the entire stem was sampled. Each sample was combined with 10 ml of 0.1% buffered peptone water and stomached for 90 seconds.

Root Analysis:

Roots were washed in distilled water, until no residual soil remains. The roots were surface sterilized by spraying with 70% ethanol solution and allowed to dry under a laminar flow hood until no visible ethanol remains. Then the root sample was combined with 10 ml of 0.1% buffered peptone water and stomached for 90 seconds.

Tomato Analysis:

Both green and red ripe tomatoes were harvested from plants. Tomatoes were surface sanitized by spraying 70% ethanol solution and then allowed to dry under a laminar flow hood, until no visible ethanol remains. The tomatoes were then placed in individual sterile stomacher bags containing 10 ml 0.1% peptone water and stomached for 90 seconds.

Analysis of homogenates:

Trial #1:

Each plant homogenate sample underwent a pre-enrichment procedure to track *S.* Newport internalized into the plant. Serial dilutions using 0.1% peptone water were plated

in duplicate onto TSA supplemented with 50ppm nalidixic acid. HEA was used for Growth Chamber 2 Early Fruit. Mid Fruit was sampled using Xylose Lysine Deoxycholate **(**XLD) agar (Becton Dickson and Company, Sparks, MD) and a T-streak method was preformed. *S*. Newport positive colonies were red with some possible black centers. Growth Chamber 1 Full Fruit and Terminal and Growth Chamber 2 Mid Fruit, Full Fruit, and Terminal plant tissues were pre-enriched using XLT-4 agar, using the T-streak methodology. Typical *S*. Newport colonies were black centered from H_2S production after 18-24 hours of incubation. All plates were incubated at 37°C for 24 hours. Potential positive *S*. Newport colonies were observed and recorded.

Trial #2:

Due to the successful prevention of background microflora growth using XLT-4 agar, plant homogenates for the second trial were sampled exclusively with XLT-4 agar. Plant homogenates were pre-enriched using the T-streak methodology and in duplicate was plated onto XLT-4 agar. The plates were incubated at 37°C for 24 hours. Presumptive positive S. Newport colonies were observed and recorded.

Enrichment procedures:

Trial #1:

To identify low levels of contamination, enrichment procedures were also used. Ten ml of Lactose broth (Becton Dickson and Company, Sparks, MD) was inoculated, with 1 ml of the homogenate, to be used as a pre-enrichment procedure. Inoculated Lactose broth was incubated at 37°C for 24 hr. One ml transfers were then made into Selenite Cysteine (Remel, Lenexa, KS) broth to begin selective enrichment. Inoculated Selenite Cysteine broth was incubated at 37°C for 24 hr, streaked onto HEA and incubated for an additional 24 hr at 37°C. Presumptive *S.* Newport colonies were identified by typical colony formation with H_2S production.

Trial #2:

Enrichments were inoculated into 10 ml Tetrathionate broth (Remel, Lenexa, KS) in 1 ml increments of the homogenate. Tetrathionate broth was incubated at 37°C for 24 hr

then streaked onto XLT-4 agar. After 24hour incubation at 37°C, the plates were identified by typical *S*. Newport colony formation.

Multiplex Polymerase Chain Reaction:

After enrichment procedures, presumptive positive colonies identified in trial #2 were confirmed using multiplex polymerase chain reaction (PCR). Each presumptive colony was grown in 10 ml TSB at 37°C for 24 hours to an approximate 10^7 optical density, before beginning. The culture was centrifuged at 7,500 x g for 10 minutes then re-suspended in sterile DI water. DNA was extracted using the Qiagen DNeasy Plant Mini Kit (Valencia, CA), per manufacturer's instructions, beginning with step 7 of the Plant Tissue Mini Protocol *(1)*. Concentration and purity of the DNA was determined using the Nanodrop® ND1000 spectrophotometer (NanoDrop Industries Inc., Wilmington, DE) by examining the Optical Density 260 (OD260) and Optical Density 280 (OD280) ratios. DNA more concentrated then 100 ng/µl was diluted using sterile DI water. A "master mix" of PCR reagents was prepared containing 0.05 µM (*gapA*, *invA*, *apeE*), purified DNA (100 ng/ µl), and 2x Taq Mastermix (New England Biolabs, Ipswich). (3). The potential positive isolates were prepared to select for the 16S rDNA gene to later send for sequencing. PCR conditions were as follows: initial denaturation at 96 °C for two minutes, followed by 35 cycles of: 96°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute and a final extension of 10 minutes at 72°C (3).

Gel Electrophoresis:

To visualize the DNA fragments, gel electrophoresis was used to separate PCR fragments by size on a 1.5% agarose gel, in 1x tris-acetate EDTA (TAE) buffer (Fisher BioReagents, Pittsburgh, PA), and a 0.5 µg/ml concentration of ethidium bromide (Fisher BioReagents, Pittsburgh, PA). Each 10µl sample was loaded into a well on the gel with Blue/ Orange 6X loading dye (Promega, Madison, WI). A 100 bp DNA ladder (Promega, Madison, WI) served as a marker. The gel was visualized using UV light and the bands were compared against a known *S.* Newport culture and a negative control sample to confirm results.

Results and Discussions:

The presence of *Salmonella enterica* serotype Newport was detected in both the tomato roots and stems but was never detected in the tomato fruit for both trial #1 and trial #2 (Table A.2, Table A.3, and Table A.4). For trial #1, 13% (12 out of 92) of negative control and 24% (22 out of 92) *S*. Newport irrigated tomato plant samples were presumptively positive for *S*. Newport (Table A.2). Of the 12 negative control samples, 67% were root samples, 17% were stem samples, and 17% were leaf samples (Table A.2). Of the 22 positive *S*. Newport irrigated samples, 55% were root samples, 32% were stem samples, and 14% were leaf samples (Table A.2).

Trial #1 tomatoes were grown under conditions and used detection methodology that possibly influenced the results. Growth Chamber 1 tomato seeds were planted in a potting mix with very high organic matter content, resulting in delayed growth from seed to three true leaf stage seedlings, possibly due to nutrients leaching from the soil. After the seeds were transplanted into un-sterilized BOJAC series soil, background microbial growth interfered with the desired detection methodology. Using TSA supplemented with 50ppm nalidixic acid, the agar plates were difficult to distinguish between *S*. Newport positive and overwhelming background growth, later identified as *Enterobacter aerogenes*. For Growth Chamber 2 Early Fruit samples, HEA was used instead of TSA-N in hopes of minimizing the background growth. Background growth decreased but potential *S*. Newport positive samples were difficult to distinguish. Growth Chamber 2 Mid Fruit was sampled onto XLT-4 agar for its increased selectivity. *S*. Newport presence or absence was detected throughout the remainder of the experiment. In both growth chambers, there was difficulty enriching for *S*. Newport using Lactose broth and Selenite Cysteine broth. All enrichment samples were *Salmonella* negative, regardless of positives that occurred on plates prior to enrichment.

In trial #2, before enrichment 19% (6 out of 32) negative control and 53% (17 out of 32) *S*. Newport irrigated tomato plant samples were positive for *S*. Newport (Table A.3). Of the 6 negative control samples, 67% were root samples and 33% were stem samples (Table A.3). Of the 17 positive *S*. Newport irrigated samples, 47% were root samples, 24% were stem samples, and 29% were leaf samples (Table A.3). The tomato plants were only sampled at 14 days after transfer and Early Fruit because additional time was needed to

make methodological improvements before the next tomato set could be planted.

After inoculation of the sample homogenate into an enriching Tetrathionate broth then streaked onto XLT-4 agar, additional trial #2 samples were potentially *S*. Newport positive (Table A.4). An additional 4 negative control, 2 root and 2 leaf samples, and 3 *S*. Newport irrigated samples, 2 stem and 1 leaf samples, showed potential *S*. Newport presence (Table A.4).

Tables and Figures:

Table A.1: Nutrient content for autoclaved and un-autoclaved BOJAC series soil. Results obtained from Virginia Tech's Soil Testing Lab.

Test	Not Autoclaved Soil	Autoclaved Soil
pH	5.99	5.99
BpH	6.17	6.18
P (ppm in soil)	200	188
K (ppm in soil)	118	125
Ca (ppm in soil)	780	681
Mg (ppm in soil)	72	69
Zn (ppm in soil)	1.7	1.3
Mn (ppm in soil)	11.3	25.8
Cu (ppm in soil)	1.5	1.2
Fe (ppm in soil)	26.0	26.2
B (ppm in soil)	0.2	0.2
Cation Exchange Capacity (meg/100g)	0.062	0.056
Acidity (meg/100g)	0.222	0.234
Base Sat (meg/100g)	0.778	0.766
Ca Sat (meg/100g)	0.633	0.608
Mg Sat (meg/100g)	0.096	0.101
K Sat (meg/100g)	0.049	0.057
Organic Matter (meg/100g)	0.014	0.014
Soluble Solids (ppm)	128.0	128.0

* There was not a high discrepancy between the autoclaved and un-autoclaved soil so the un-autoclaved BOJAC series soil was used throughout the experiment.

Table A.2: Number of tomato plant samples (roots (R), stems (S), leaves (L), and fruit (T1 and T2)) from trial #1, presumptively positive for *S. enterica* serotype Newport (prior to enrichment of the homogenate) in plants irrigated with water (control) and *S*. Newport at different growth cycle stages grown under controlled environmental conditions.

	Irrigated with Water (n=4)					**Irrigated with *S*. Newport (n=4)**				
	R	**S**	**L**	**T1**	**T2**	**R**	**S**	**L**	**T1**	**T2**
14 Days	4	2	2	N/A	N/A	4	3	3	N/A	N/A
Early Fruit	2	0	0	0	0	1	1	0	0	0
Mid Fruit	2	0	0	0	0	2	1	0	0	0
Full Fruit	0	0	0	0	0	3	1	0	0	0
Terminal	0	0	0	0	0	2	1	0	0	0

Table A.3: Number of tomato plant samples (roots (R), stems (S), leaves (L), and fruit (T1 and T2)) from trial #2, presumptively positive for *S. enterica* serotype Newport (prior to enrichment of homogenate) in plants irrigated with water (control) and *S.* Newport at different growth cycle stages grown under controlled environmental conditions.

	Irrigated with Water (n=4)					**Irrigated with *S.* Newport (n=4)**				
	R	**S**	**L**	**T1**	**T2**	**R**	**S**	**L**	**T1**	**T2**
14 Days	1	2	0	N/A	N/A	4	2	3	N/A	N/A
Early Fruit	3	0	0	0	0	4	2	2	0	0

Table A.4: Number tomato plant samples (roots (R), stems (S), leaves (L), and fruit (T1 and T2)) from trial #2 presumptively positive for the presence of *S. enterica* serotype Newport (after enrichment) in plants irrigated with water (control) and *S.* Newport at different growth cycle stages grown under controlled environmental conditions.

	Irrigated with Water (n=4)					**Irrigated with *S.* Newport (n=4)**				
	R	**S**	**L**	**T1**	**T2**	**R**	**S**	**L**	**T1**	**T2**
14 Days	2	2	2	N/A	N/A	4	4	4	N/A	N/A
Early Fruit	4	0	0	0	0	4	2	2	0	0

Figure A.1: 16S rDNA Multiplex PCR results of control root (Lane 7, Lane 11), contaminated root (Lane 10, Lane 17), contaminated stem (Lane 5, Lane 13), control stem (Lane 8, Lane 12), control leaf (Lane 2, Lane 3), contaminated leaf (Lane 4, Lane 6, Lane 15, Lane 16), positive *S*. Newport control (Lane 14), negative control (Lane 9), and Promega 100 bp genomic ladder (Lane 1, Lane 18) to identify *S*. Newport from presumptive *Salmonella* positive Growth Chamber 3 and Growth Chamber 4 plate isolates after enrichment.

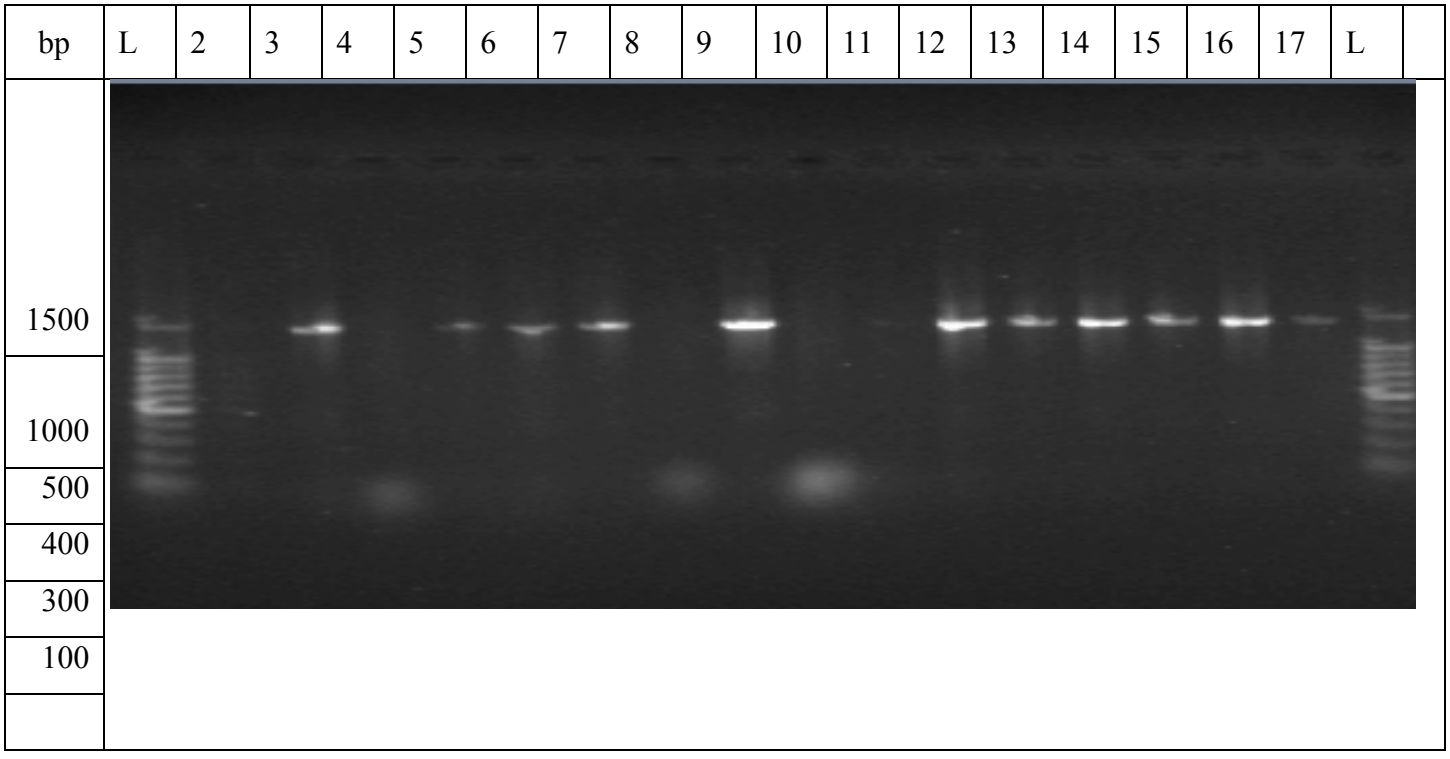
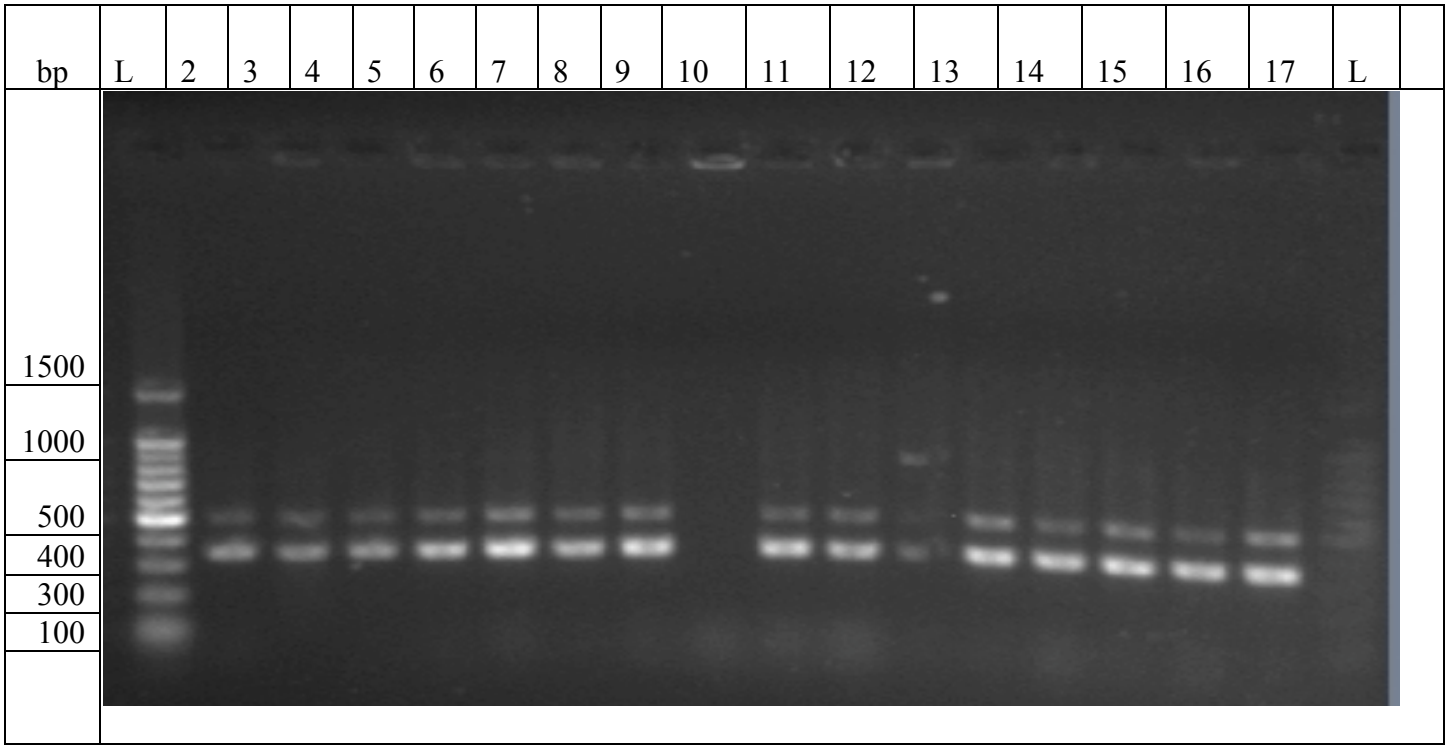


Figure A.2: Multiplex PCR results (*invA*, *gapA*, and *apeE)* confirming presumptive positive S. enterica isolate from control root (Lane 7, Lane 11), contaminated root (Lane 10, Lane 17), contaminated stem (Lane 5, Lane 13), control stem (Lane 8, Lane 12), control leaf (Lane 2, Lane 3), contaminated leaf (Lane 4, Lane 6, Lane 15, Lane 16). Gel includes positive *S.* Newport control (Lane 14), negative control (Lane 9), and Promega 100 bp genomic ladder (Lane 1, Lane 18).



1. Qiagin. 2006. DNeasy Plant Handbook.