

Role of airborne soil particulate in transfer of *Salmonella* spp. to tomato blossoms and consequential fruit contamination

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ABSTRACT

Contaminated tomatoes have become a commonly implicated vehicle of *Salmonella* outbreaks. Exposure of tomatoes to pathogen could occur in the field. Blossom inoculation with *Salmonella* can result in contaminated fruit but natural routes of blossom contamination are not well known. Salmonellae have been known to survive in agricultural soil. Since dispersal of soil particulate by wind is a common phenomenon, the potential of airborne soil particulate as a vehicle of *Salmonella* contamination in tomato blossoms was examined.

It was determined that *Salmonella* enterica serotype Anatum, Baildon, Braenderup, Montevideo, Newport, Javiana had similar survival patterns in both soil and water. At the end of 40 days, populations of *Salmonella* in soil dropped by 2.59 log CFU/g and 5.11 log CFU/g when enumerated on Tryptic Soy Agar Yeast Extract (TSAYE) and xylose lysine Tergitol 4 (XLT4) agar respectively. *Salmonella* populations in water reduced by 2.55 log CFU/ml (TSAYE, enumeration) and 2.94 log CFU/ml (XLT4, enumeration). Blossom to fruit formation takes 20-30 days in tomatoes hence the introduction or presence of the pathogen in agricultural soil and water could increase risk of blossom contamination. Also, it was determined that all *Salmonella* serotypes tested were capable of biofilm production on glass coverslips and quartz particles. Biofilm based attachment of *Salmonella* to sand might aid in its dispersal.

To visualize transfer of pathogen from soil particulate to blossom in real-time, bioluminescent *S. Baildon*, *S. Braenderup*, *S. Newport*, *S. Javiana* and *S. Anatum* were created.

Heat shock procedure was developed to improve electrotransformation efficiency in *Salmonella*. Transformed strains were compared for bioluminescence production and plasmid stability. *S. Newport* had the best bioluminescence properties but no difference was observed between strains for plasmid stability.

Imaging of soil particulate - *S. Newport* mixture inoculated blossoms, indicated that the event led to pathogen transfer to blossom. It was also determined that *S. Newport* – soil particulate contaminated blossoms developed into fruits that were positive for *S. Newport*. *S. Newport* presence in blossom, fruit surface and internal tissue indicates that contaminated soil particulate could serve as a vehicle of tomato contamination.

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DEDICATION

I dedicate this work to my parents, Mrs. Padmini Dev Kumar and Mr. Dev Kumar Bala.

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CHAPTER 1. INTRODUCTION AND JUSTIFICATION

Nearly half of all produce associated illnesses caused by bacteria are attributed to *Salmonella* spp. Salmonellae are a leading cause of foodborne illness in the United States, infecting 1.4 million people and causing fiscal losses of 2.7 billion dollars due to lost productivity and medical expenses in 2010 (1). In 2009, 40,828 clinical confirmed human isolates of *Salmonella* were reported to the CDC (5). Tomatoes have become the most frequently implicated vehicle in cases of produce associated salmonellosis (4).

Over a dozen *Salmonella* outbreaks have been associated with the consumption of tomatoes since 1990. About 5 billion pounds of fresh market tomatoes are consumed annually in the United States (9) hence *Salmonella* contaminated fruit can result in widespread illness and economic loss. Fresh tomatoes are often consumed raw with minimal processing or heat treatment; consequentially, there is an increased risk of pathogen transmission from contaminated product. Fresh fruit and produce could be contaminated by spoilage or pathogenic organisms during pre-harvest and post-harvest procedures (8). It has also been demonstrated that produce might be contaminated during production in the field or in the packing facility (6, 7). Understanding routes through which pathogen exposure occurs in produce is important to develop effective measures to prevent contamination.

Surface contamination and presence of *Salmonella* in internal tissue of tomato fruits has been demonstrated by facilitating contact of fruit with contaminated soil (8). Tomatoes grown in fields where contaminated irrigation water was used were implicated in two multistate outbreaks (7), though the use of *Salmonella* contaminated irrigation water in laboratory based experiments

did not lead to presence of pathogen in surface sterilized fruit tissue (11). Understanding the potential of soil as a vehicle of *Salmonella* and the role of winds in its dispersal could help understand on-field risks better.

The brushing of blossoms with *Salmonella* has resulted in the pathogen being present in fruit tissue, indicating that blossoms might be a potential site of pathogen entry. Natural routes of blossom contamination need to be determined. Dust is defined as fine particulate matter removed from land surface due to winds (13). A strong correlation was found between *Salmonella* spp. presence and airborne particulates in a study conducted by Lues et al. at a high throughput poultry slaughter facility indicating that dust could have an influence on the dispersal of *Salmonella* spp. (10). Thus it is important to determine if wind-forced movement of *Salmonella* contaminated soil particles could result in pathogen transport to tomato blossoms in agricultural fields.

Salmonella spp. have been known to persist in the environment (3, 7) hence it is important to understand population dynamics of tomato outbreak associated *Salmonella* during survival in soil to avoid pathogen transfer to crops. Biofilms have been known to aid in attachment and environmental persistence (12) but their role in attachment to soil has not been explored. The attachment of *Salmonella* serotypes to soil particulate could provide the pathogen a means for wind associated transfer of pathogen in field. Visualization of *Salmonella* dispersal in real-time on blossom tissue and could help in better understanding of tomato contamination.

Biophotonic imaging is a non-invasive, non-toxic method of imaging in real-time using visible light that is emitted during bioluminescence or through excitation (2). Bioluminescence has many applications such as understanding bacterial pathogenesis, monitoring gene expression

non-invasively, monitoring hygiene and food safety research (2). Biophotonic imaging can be used to study *Salmonella* spp. attachment to blossoms. *Salmonella* cells require to be transformed with a plasmid containing the *lux* operon for the development of bioluminescent *Salmonella* serotypes.

Salmonella is not naturally competent and are endowed with restriction enzymes that reject foreign DNA. Difference between bioluminescence expression could occur amongst serotypes. Overcoming restriction associated barriers and determining differences in luminescence expression could help us develop a robust tool for imaging pathogen interaction with plant tissue.

The objectives of this research project are to a) determine survival patterns of tomato outbreak associated *Salmonella* serotypes in soil and water b) test *Salmonella* serotypes for their ability to form biofilms c) develop bioluminescent *Salmonella* serotypes for real time imaging studies d) determine if dust mediated contamination of tomato blossoms can lead to contamination of fruit. The accomplishment of these objectives will help us determine the potential of soil particulate as a risk factor for *Salmonella* contamination of tomatoes.

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CHAPTER 2. LITERATURE REVIEW

Scope of the problem

Consumption of a variety of produce has become an integral part of the American diet. Awareness of the potential of produce consumption in reducing risk of developing cancers and cardiovascular disease has resulted in an increase in production and sales of a variety of produce in recent years (15). The annual average weight of fresh produce consumed in 1997-99 rose by 25% compared to 1977-1979 (15, 92). Sales of salad mixes in 1994 burgeoned to \$507 million from \$167.5 million in 1991 according to the International Fresh Cut Produce association, Arlington, VA (53). Increase in demand for a variety of produce could be one of the factors that influenced the increase in produce prices (53).

Since most produce is consumed raw with minimal processing or heat treatment, there is an increased risk of pathogen transmission from contaminated product. Fresh fruit and produce could be contaminated by spoilage or pathogenic organisms during pre-harvest and post-harvest procedures (49). It has also been indicated that the produce might be contaminated during production in the field or in the packing house (18, 29). There has been an increase in produce related outbreaks. Between 1973 and 1987 it was reported that fruit and vegetables were associated with only 2% of foodborne disease outbreaks in the United States (7). The percentage of outbreaks associated with foodborne pathogen contaminated produce increased to 6% by the 1990's and is currently at 13% (16, 44)

The increased demand for fresh produce and the challenges in cultivation due to seasonal variation has led to increased importation. Fresh produce demand has also led to changes in agricultural processing and distribution (15). Both imported and locally grown produce might be contaminated with pathogens. Research has shown that the quality of produce cultivated in

Mexico is similar to produce cultivated in the United States (62). Outbreaks have been associated both with locally grown and internationally acquired produce (20, 91). Challenges associated with contaminated produce that has been imported include enhanced difficulty in conducting traceback investigations and the introduction of rare or unfamiliar pathogenic strains into the food chain (20, 91).

The use of standardized sub-typing technique such as Pulse Field Gel Electrophoresis has allowed laboratories in all 50 states in the US to submit PFGE patterns electronically to PulseNet. This has improved accuracy of source detection and traceback operations, even for cases that span multiple states and involve multiple serotypes (43). Thus factors such as higher uncooked produce consumption, increased importation rates, frequent handling, extensive distribution procedures and sensitive detection techniques could have contributed to the increase in percentage of foodborne outbreaks associated with produce consumption (15, 17).

Produce can be contaminated by viruses, parasites and bacteria. Viruses such as Norovirus and Hepatitis A can be transferred on to produce through handling and other processes. According to Sivapalasingam et al. viruses contributed to 20% of the outbreaks associated with produce between 1973-1997 in the United States (101). Parasites such as *Cyclospora* and *Cryptosporidium* spp. have been associated with produce associated outbreaks and contributed to 16% of all produce associated infections reported in the US from 1973 to 1997. Bacterial agents were the major pathogens responsible for produce borne illnesses during the same time span contributing to 60% of all produce associated illnesses (15, 101). Nearly half of these produce associated illnesses are attributed to *Salmonella* spp. Salmonellae are the leading cause of bacterial foodborne illness in the United States infecting 1.4 million people and causing fiscal losses of \$ 2.7 billion dollars due to lost productivity and medical expenses in 2010 (1). In 2009,

40,828 isolates from clinically confirmed cases were reported to the CDC (22). Over a dozen *Salmonella* outbreaks have been associated with the consumption of tomatoes since 1990. Recently, tomatoes have become the most implicated vehicle for produce associated salmonellosis (12).

***Salmonella*- The pathogen, clinical outcome of disease and impact**

Salmonella are a Gram negative, orally acquired intracellular facultative anaerobic bacteria resulting in 1.3 billion infections worldwide. In the United States, approximately a million infections are caused by *Salmonella* serotypes each year (98). The genus *Salmonella* has contains 2 species, *S. enterica* and *S. bongori*. *S. enterica* is further divided into *Salmonella enterica* subsp. *enterica* (subsp. I), *Salmonella enterica* subsp. *salamae* (subsp. II), *Salmonella enterica* subsp. *arizonae* (subsp. IIIa), *Salmonella enterica* subsp. *diarizonae* (subsp. IIIb), *Salmonella enterica* subsp. *houtenae* (subsp. IV), and *Salmonella enterica* subsp. *indica* (subsp. VI). (18). The subspecies are further divided into more than 2500 serovars based on flagellar, carbohydrate and lipopolysaccharide (LPS) structure (28).

The spectrum of clinical manifestations of *Salmonella* infections includes gastroenteritis, Typhoid fever, bacteremia and focal infections. Typhoid fever is caused by *Salmonella enterica* serovar Typhi, a pathogen of rare occurrence in developed countries but common in the developing world. The transmission of *S. Typhi* is usually human to human and is associated with fecal contaminated water. *S. Typhi* is considered a human pathogen and there are no known animal reservoirs (39, 89).

Foodborne *Salmonella* or non-typhoid *Salmonella* usually cause mild gastroenteritis within 6-72 hours after consumption of contaminated food. The symptoms caused by foodborne *Salmonella* consist of diarrhea, abdominal cramps, vomiting and fever. The severity of the

disease depends on the dose and virulence of involved serotype and the condition of the host (64). In certain cases infections could result in septicemia and invasive infections of organs and tissues. *Salmonella* infections occur mostly through the consumption of contaminated food and water in humans. Routes of infections such as tonsils and the respiratory system are also potential sites of infection in cattle and swine(116).

Mechanism of infection: Fecal-oral transmission is the primary route of *Salmonella* infections. The number of *Salmonella* cells required to cause infection is highly variable and can range from 30 to a billion cells. This could depend on the serotype, condition of the host species and the type of food in which the organism is present (64). Foods with high fat content could result in infection with lower doses of the pathogen (39, 82).

Upon entry into host through a contaminated food source, *Salmonella* cells have to survive the low acid environment in the host's stomach. The Acid Tolerance Response proteins help in low pH environment survival. These include RpoS and PhoPQ for regulation of tolerance in inorganic acid. Fur and RpoS proteins are responsible tolerance regulation in low pH environments caused by organic acids (39).

Salmonella serotypes often cause infection in animal hosts by inducing their own uptake into intestinal epithelial cells. Gastroenteritis causing serotypes induce an inflammatory and secretory response while serotypes associated with systematic infection survive and replicate in mononuclear phagocytes (29). *Salmonella* spp. express many fimbriae and pili that help them adhere to intestinal epithelial cells. The Type iii secretion system (T3SS) consists of intricate structures that the pathogen has evolved to promote endothelial uptake and invasion. The base structure of the T3SS, spans the cell wall and cell membrane. A needle like structure protrudes from the base and interacts with the host cell. Within the base and needle like structure is an

inner rod that forms the conduit between bacterial cytoplasm and host cell membrane. The needle like structure interacts with a “translocation complex” in the host cell membrane to promote transfer of effector proteins into host cell cytosol. Proteins making up the translocation complex are initial effector proteins of the T3SS produced by *Salmonella*. The needle complex interacts with the translocation complex to allow delivery of bacterial proteins into the host cytosol. Adherence of bacteria to apical epithelial surface results in profound cytoskeletal rearrangements resulting in disruption of normal epithelial brush border. Consequentially membrane ruffling is induced resulting in envelopment of bacterial cells in vesicles. A secretory response in the intestinal epithelium is initiated by serotypes associated with gastrointestinal onset of disease. This results in neutrophil migration in the intestinal lumen. The *Salmonella* Pathogenicity island 1 (SPI1) translocated T3SS proteins are responsible for the onset of diarrhea. The SopB proteins appear to play important roles in activation of secretory pathways and neutrophil attraction, resulting in increased inflammation and an alteration of ionic balance (40, 85, 113). The T3SS proteins of *Salmonella* Pathogenicity island 2 (SPI2) are responsible for survival inside macrophages (4). New SPI's are being identified with size, structure and functions varying between *Salmonella* subspecies (77) . *Salmonella* pathogenesis is a complex multifactorial phenomenon involving complex manipulation of the host's immune system (28). While most cases are self-limiting, the impact of foodborne salmonellosis is tremendous. Traditionally *Salmonella* outbreaks are caused by contaminated poultry, egg, beef and dairy products (45). Recently produce has strongly contributed to the number of foodborne infections caused by salmonellosis (101).

In 1999, Mead et al., estimated that 76 million foodborne illnesses, 325,000 hospitalizations and 5,000 mortalities occur in the United States (US) every year (75). The

estimates in 2011 according to the CDC reveal a significant decrease in these numbers. In 2011, it was estimated that there were 48 million foodborne illnesses, 128,000 hospitalizations and 3,000 deaths each year in the US (97). Though these figures indicate a significant decrease in foodborne diseases it has been indicated that the 2011 study cannot be compared to the 1999 estimates because of differences in analytical techniques (87). Data from estimates through surveys conducted by the Foodborne Disease Active Surveillance Network (FoodNet) of the CDC's Emerging Infections Program indicates a less dramatic drop in foodborne illness associated pathogens such as *Salmonella*. In 2009, there was a 10% drop in the incidence of infections caused by *Salmonella* serotypes in comparison to the period from 1996 to 1998. Certain *Salmonella* serotypes such as *S. Typhimurium*, *S. Agona*, *S. Heidelberg* and *S. Thompson* showed a decrease in incidence while serotypes such as *S. Javiana* and *S. Newport* demonstrated significant increases in occurrence (8). This indicates that while control measures to prevent foodborne infections have been effective, the threat of infrequently encountered serotypes gaining dominance in the future exists. Monitoring emerging serotypes, determining ecological niches of these serotypes, understanding their host interaction strategies and survival in the environment is an important step in reducing risk associated with emerging *Salmonella* serotypes.

Tomatoes

Tomato (*Lycopersicon esculentum*) belongs to the nightshade family (*Solanaceae*). The *Solanaceae* family also includes crops like chilli, bell pepper and eggplant. The tomato is a major horticultural crop with an estimated global production of over 120 million metric tons according to the FAO in 2007. Studies have shown that tomato consumption has the potential to reduce risk of certain types of cancer, cardiovascular diseases and age-related macular

degeneration (35, 44). Approximately 5 billion pounds (2 267 961.85 metric ton) of fresh market tomatoes are eaten annually in the United States (52). The United States is the second major producer of tomatoes and leads the world as the exporter of processed tomato products (56).

Tomato fruits are classified as classic round, cherry cocktail, plum and baby plum, beefsteak and vine or truss tomatoes. Based on use, tomatoes are termed as “Fresh Market” and “Processing”. Fruits are also categorized based on their ripeness and this determines when fruits are picked. Ripening is based on color, texture, flavors and aromas (46). The different classifications based on ripeness for tomatoes include the immature, mature green, breaker, turning, pink, light red and red ripe. Tomatoes for processing are harvested during the red-ripe stage and transported immediately for processing. Tomatoes for the “fresh market” are harvested between the mature green and red ripe stages, with the breaker stage being the most common stage of development for harvesting. During the breaker stage there is a break from green to yellow, pink or red on the blossom end of the fruit. The pH of tomato fruit ranges around 3.4 to 4.8, and depends on the stage of fruit maturity (63, 95).

States primarily responsible for the production of “Fresh Market” tomatoes are Florida, California, Ohio, Georgia and Virginia. Leading states that produce fruit for “Processing” are California, Ohio, Indiana and Michigan (56, 63).

Tomatoes can be machine-harvested or hand-harvested. Tomatoes cultivated for processing are usually machine harvested while “fresh market” tomatoes are usually hand-harvested. After being hand-picked and collected in bins or baskets they are transported to a packing shed. On reaching the packing shed, fruit can be flumed out with chlorinated water or bins and boxes are unloaded by hand into water or on to a padded table. Care is taken, to prevent dumping fruit into cold water to eliminate field heat and are instead dumped into water with

temperature a few degrees higher than the fruit. This is done to prevent entry of water and pathogen uptake due to water absorption into the fruit resulting from the temperature difference. Pre-sizing and inspection may be followed by a waxing process. Following which the tomatoes are sorted by color. Vine ripe tomatoes are separated by their size and graded for quality and then undergo packing and cooling before shipment to market or retail stores. Mature green tomatoes are also sorted by size and by the use of sizing belts and are graded on quality following which they undergo packing and cooling. The fruit is treated for ripening before or after shipment to the wholesaler, after which they reach the market place (95).

Salmonellosis associated with tomato consumption

For over 50 years, salmonellosis resulting from consumption of contaminated fruit has been reported (53). Fruits such as watermelon and cantaloupes have been determined as sources of *Salmonella* infections. Though traditionally associated with undercooked poultry eggs, beef and certain dairy products, *Salmonella* outbreaks in the recent past have been associated with produce at higher frequency. Since 1990 over a dozen *Salmonella* outbreaks have been reported in association with consumption of raw uncooked tomatoes, more than any other types of produce (Table 2.1) (12, 15, 53).

Several factors such as, higher produce consumption, more sensitive detection techniques and improvements in disease reporting have all been attributed to the increase in reported outbreaks (36). Relatively rare serotypes have been associated with some produce related outbreaks. Every year more than a 100,000 estimated cases of infection are caused by *S. Newport* each year, while *S. Braenderup* has been previously associated with chicken, jelly and eggs and have been responsible for 1.6% of the total number of *Salmonella* associated illnesses reported in 2003 tomatoes (47, 52). This could indicate that new farming, produce processing

and distribution might practices might be increasing risk of *Salmonella* contamination of tomatoes. Traceback efforts from several tomato associated *Salmonella* outbreaks reveal that the farm and packing house might serve as sources of contamination. Different sources of contamination could exist. These include handling of raw tomatoes during harvesting, surface contamination from contact surface such as bins, low water temperature of the dump tank and contamination while slicing (47, 52). Contamination sources could also include soil, water, manure, animals, birds, and insects. Genetic analysis and phyllosphere- rhizoplane persistence studies of *Salmonella* serotypes indicate better fitness of certain serotypes over others. Shi et al. showed that members of group C serovars were more proficient at plant colonization than serovars associated mostly with poultry (group D serovars) (53). There is considerable economic loss due to ill health and recall of contaminated product resulting from the presence of *Salmonella* serotypes in produce such as tomatoes.

The consumption of uncooked fresh tomatoes packaged in a South Carolina packing house was responsible for two separate outbreaks in the year 1990 and 1993. The packing house received tomatoes from 12 individual growers and operated seasonally for a 3-4 week period in June and July of 1993.

It was determined through data from laboratory based surveillance of four states that a multi-state outbreak involving four states had occurred during two separate years. The outbreak in 1990 was caused by *S. Javiana* and had resulted in 176 cases. *S. Montevideo* was responsible for 100 cases of salmonellosis in 1993 (Table 2.1). Most of the individuals implicated in the outbreak had consumed tomatoes from restaurants. Some of them had consumed tomatoes procured from grocery stores. A traceback investigation revealed that the farms used drip irrigation. Well water and pond water were used as water sources. Water was not tested for

microbiological quality during the investigation because production had stopped. Water in the packing house used to wash tomatoes was chlorinated with chlorine gas as a disinfection measure. The multistate outbreak was a one of the first since 1990 of a growing trend in *Salmonella* associated tomato outbreaks (54). It should be noted that two different serotypes were responsible for the outbreaks though the tomatoes were packaged in the same packing house (Table 2.1). This could indicate different sources or prevalence of multiple serotypes in the farm environment or in the packing house. The traceback study was not able to determine the source of the pathogen, though water in the dump tank was suspected. The airspaces in tomatoes can constrict when tomatoes are dumped into cold water due to a difference in temperature. This could result in a vacuum due to decrease in airspace volume and result in the drawing of water and pathogenic organisms that might be present in the dump tank water, into the fruit (32).

In 1998/1999, 86 individuals from 8 different states were infected with *S. Baildon* (Table 2.1). *S. Baildon* is considered as a rare serotype. Through case control studies it was determined that “restaurant served” raw tomatoes was the source of the serotype and contamination of tomatoes likely occurred in the farm or the packing house. Two grower/packing cooperatives were determined as the possible suppliers of contaminated tomatoes. The operating packing facility that was inspected reported using chlorinating water and monitoring temperature of water in the dump tanks as methods of hazard control. The tomato dicing operation stored mechanically packaged diced tomatoes in 5 pound trays at 4.4°C. Chlorine level of up to 130 ppm was maintained in the facility, according to the processor. Higher levels of chlorine would be required to eliminate *Salmonella* Baildon from diced fruit and from crevices on the surface of whole fruit. The efficacy of chlorination depends on the pH of wash or dump tank water,

chlorine concentration, water temperature, amount of organic matter present, exposure time, location and population density of *Salmonella* (32, 118).

Water temperature difference between fruit and wash water could have also contributed to the presence of the pathogen in fruit tissue. Pooling of cut fruit could have also contributed to the distribution of the pathogen over the fruit tissue (32). The traceback results from these outbreaks indicate better control measures are required during operations in the packaging facility and farms.

In 2000, a multistate tomato associated outbreak was caused by *S. Thompson*. Forty two individuals were affected by the outbreak but the source of the serotype was not tracked (53). *Salmonella* serotypes Newport and Javiana were responsible for tomato associated outbreaks in 2002 (Table 2.1). The *S. Javiana*-tomato associated outbreak occurred in a theme park where the 2002 US Transplant Games that included 1,500 organ donors. Pre-packaged diced Roma tomatoes were implicated as the cause of the outbreak. Twelve people who had visited the park but had not participated in the games were also affected by illness resulting from contaminated fruit (109).

While serotypes such as *S. Baildon* are considered rare, *S. Newport* has been reported to cause an estimated 100,000 infections in the United States on an annual basis (47). It has been associated with the consumption of beef and horse meat and produce including tomatoes. An outbreak in 2002, associated with the consumption of raw uncooked tomatoes grown and packed in the Eastern Shore of Virginia affected 510 people in 26 states (66). An outbreak in 2005 was also caused by *S. Newport*, affecting 72 people in 16 states. It was observed that both outbreaks had strong commonalities such as a similar strain, PFGE pattern, case-patient distribution (all but one of the patients resided in states on the east side of the Mississippi) and association with

tomatoes. The traceback study revealed that “red round” tomatoes served in the restaurants where the infected patients had eaten were supplied from a farm/ packing house in the Eastern Shore in Virginia. Environmental sampling of the farm revealed that the same strain isolated in the outbreaks of 2002 and 2005, was also isolated from the irrigation pond water sample. The irrigation sample entered the soil bed and did not come in direct contact with fruit. It was also observed that pond water in the farm was positive for *S. Newport* and *S. Javiana* of another PFGE pattern during an inspection of the farm at a different time period. These observations indicate that the well water used for irrigation could potentially have been contaminated due to soil seepage and cross contamination of pipes supplying water. Geese and turtles were also observed in the farm location indicating other possible sources of *Salmonella* and routes of contamination (47).

Three outbreaks occurred in the summer of 2004 resulting in 561 people got infected from 16 states and Canada. One of the multi-state outbreaks was caused by a relatively rare serotype, *S. Braenderup* resulting in 125 confirmed cases. Another multi-state outbreak resulting in 429 cases occurred between June 25 and July 14 of 2004 and was caused by multiple *Salmonella* serotypes (*S. Javiana*, *S. Typhimurium*, *S. Anatum*, *S. Thompson*, *S. Muenchen* and Group D untypable). Seven individuals were infected in the same summer during an outbreak in Canada that was caused by *S. Newport*. All outbreaks were linked to the consumption of Roma tomatoes(5).

The *S. Newport* outbreak in Canada was associated with the consumption of Roma tomatoes though no case control study was performed to confirm this. The outbreak caused by *S. Braenderup* was also associated with Roma tomatoes, served by two restaurants that had the same supplier. For the concurrent outbreaks caused by *Salmonella Newport* and *Salmonella*

Braenderup, the same packing house was suspected for the contaminated fruit through traceback efforts. Environmental investigations of the suspected farms and packing houses did not indicate any sources of contamination. It should be noted that only one packing house and associated farm were in operation during inspection.(5, 52)

The multi-serotype outbreak of 2004 was linked to pre-sliced tomatoes that had been procured by a delicatessen chain from a single processor. Traceback efforts led to the tracking of the tomato slicing facility. Upon inspection of the facility while in active operation, no source of contamination was identified (52). It has been observed that slicing could increase pre-existing contamination of tomatoes by transferring surface located bacteria to internal tissues of fruit possibly by the mechanical action of the knife (32).

In 2005-2006, four multi-state outbreaks associated with the consumption of *Salmonella* contaminated tomatoes were observed (Table 2.1). *Salmonella* serotypes Newport, Braenderup and Typhimurium were responsible for contamination of the fruit. The outbreaks were large, affecting 459 individuals across 21 states.

Between July to November, 72 *S.* Newport isolates with indistinguishable Pulse-Field Gel Electrophoresis (PFGE) patterns were identified. Case control studies indicated that consumption of large red round tomatoes at restaurants was responsible for disease though no single restaurant was associated with the contaminated tomatoes. Traceback efforts indicated that two farms on the Eastern Shore of Virginia were the source of the tomatoes. Environmental analysis showed that pond water used to irrigate the fields was contaminated with *S.* Newport, the same pulsotype that was implicated in a previous outbreak in 2002 (47) (7).

The same strain of *Salmonella* Newport (July to November 2008) was responsible for 115 culture confirmed cases in an outbreak spanning 19 states. No single restaurant was implicated and the source of the farm was not determined(7).

Roma tomatoes contaminated with *S. Braenderup* (November to December 2005), served in a restaurant chain were responsible for 82 culture confirmed cases. The implicated tomatoes had been grown in Florida and had been packaged in Kentucky. Environmental investigation revealed that there were multiple sources of *Salmonella* contamination such as animal reservoirs, *Salmonella* harboring drainage ditch water and animal feces from around the field. Multiple serotypes of *Salmonella* were isolated from the field, though these were different from the outbreak strain(7).

Between September and October 2006, *Salmonella* Typhimurium associated with the consumption of raw, Red Round tomatoes served in restaurants was responsible for 190 culture positive cases. The outbreak spanned 21 states in the US and also Canada. A single packing house in Ohio was traced back. The packing house was supplied with tomatoes from 25 fields in nearby counties. The end of the growing season, resulted in the investigation getting deferred(7).

The Foodborne Outbreak Database of the CDC reports that sixteen individuals in a nursing home were ill after an outbreak caused by *S. Berta* (Table 2.1). The outbreak occurred in January of 2006 and tomatoes were confirmed as the outbreak vehicle (53).

Raw whole tomatoes and cut packaged tomatoes served in restaurants or brought from stores have been involved in outbreaks. It is estimated that under reporting of illnesses due to *Salmonella* exist and only 1 out of 38 cases of salmonellosis is reported to health authorities. This indicates that the number of people affected by tomato related outbreaks could be much higher than the number of reported cases (111). Outbreaks associated with *Salmonella* could

affect individuals from various states and can also span other countries. Multiple serotypes of *Salmonella* could also be involved in outbreaks making traceback efforts more challenging (53). Environmental persistence of *Salmonella* serotypes could be an issue in farms. Outbreaks involving the same serotype and PFGE pattern can span over many years. Understanding *Salmonella* attachment and persistence on different components on fields such as soil, water, manure and dust could help determine risks on the field that could lead to contaminated fruit.

Table 2.1. Tomato-associated *Salmonella* outbreaks by year and serotype(s) involved

Year	<i>Salmonella</i> serotype	Reference
1990	<i>Salmonella</i> Javiana	(53)
1993	<i>Salmonella</i> Montevideo	(84, 118)
1998-99	<i>Salmonella</i> Baildon	(32)
2000	<i>Salmonella</i> Thompson	(53)
2002	<i>Salmonella</i> Javiana	(109)
	<i>Salmonella</i> Newport	(47)
2004	<i>Salmonella</i> Jaivana	(6)
	<i>Salmonella</i> Typhimurium	
	<i>Salmonella</i> Anatum	
	<i>Salmonella</i> Thompson	
	<i>Salmonella</i> Muenchen	
	<i>Salmonella</i> Group D(untypable)	
	<i>Salmonella</i> Jaivana	(6)
	<i>Salmonella</i> Braenderup	(6)
2005	<i>Salmonella</i> Braenderup	(52)

	<i>Salmonella</i> Newport	(47)
2006	<i>Salmonella</i> Newport	(7)
	<i>Salmonella</i> Typhimurium	(7)
	<i>Salmonella</i> Berta	(53)

Survival of *Salmonella* serotypes in tomato

The consumption of raw tomatoes has been associated with outbreaks of *Salmonella*. Though salmonellae do not produce proteinases and hemicellulases unlike closely related members associated to plants, their ability to acquire simpler carbon sources might help them survive inside plant tissues. The presence of nutrients such as simple sugars, sugar alcohols, organic acids, fatty acids and amino acids might have the potential to aid survival of *Salmonella* serotypes in fruit tissue (21, 74, 83).

Certain *Salmonella* serotypes are more adapted for survival and persistence in tomatoes and hence might be more commonly associated with outbreaks (5). Guo et al. studied the fate of salmonellae after injection of the pathogen into the stems or by brushing of the flower. Of the five serotypes used they found *S. Montevideo* to be the most persistent as it was isolated from tomatoes 49 days after inoculation. *S. Montevideo* was also the most persistent pathogen in the post-harvest environment indicating tomato contamination could occur both during pre and post-harvest production processes. *S. Poona* was found to be the most dominant as it was found in 5 of the 11 tomatoes tested (49). While it has been shown that certain serotypes are more adapted to survival in fruit tissue, the possibility of competitive dominance between serotypes might occur when a cocktail of serotypes is used, as in the experiment by Guo et al (100). Experiments by Shi et al. revealed that growth of *Salmonella* on ripened fruit depended on the

serovar the fruit was inoculated with though a diverse range of *Salmonella* can establish themselves within or on tomato surface. Their experiments also demonstrated the persistence of *Salmonella* Montevideo as the serotype was isolated from 95% of the fruits screened (100). These experiments indicate that Salmonellae are adapted for survival in the fruit, though the range of serovars might be narrow, contamination could occur before and after harvest.

Noel et al. studied the responses caused by the variety of tomato and the ripeness of fruit in *Salmonella* enterica using *In vivo* expression technology (IVET). *Salmonella* Typhimurium 14028 was compared to outbreak associated serovars from the Eastern Shore of Virginia (*S.* Newport, *S.* Braenderup, *S.* Javiana, *S.* Montevideo) for its proliferation inside red ripe tomatoes. It was found that *S.* Typhimurium grew to the same cell densities as the outbreak associated strains in the tomatoes. The strain was thus used as a model for studies in tomatoes. They found that gene expression in *Salmonella* was determined but the maturity of fruit and variety of the cultivar. Presence of *S.* Typhimurium in ripe tomatoes resulted to a strong reduction in the resolution of RIVET (recombination - based *in vivo* expression technology) reporter in the promoter adjacent to *fadH*, a gene coding an enzyme for the metabolism of unsaturated fatty acids with double bonds at even carbon positions. The presence of *S.* Typhimurium in immature tomatoes led to the upregulation of the *fadH* promoter. There was a maximum expression of *cysB*, a gene associated with antibiotic resistance, in the fruit of cultivar Hawaii 7997. Cultivar Hawaii 7997 is resistant to the vascular pathogen *Ralstonia solanacearum*, while cultivars that did not promote higher expression of *cysB* were universally susceptible to bacterial and fungal pathogens. This indicates that expression of certain genes might also be cultivar dependent (83).

Bacterial polymers and aggregative fimbriae may be involved in the attachment of *Salmonella* to plant seedlings (10). Survival in different parts of the plant like seeds, leaves,

roots, fruit surface and pulp might each present a unique challenge to the pathogen. Hence different plant tissues may elicit unique attachment and survival strategies from the pathogen. Enteric pathogens such as *Salmonella* that have transient interactions with produce such as may use different strategies for survival in comparison to plant pathogens. Noel et al. reported that about 5% overlap might occur between promoters induced in tomatoes and those induced during the infection of macrophages (106).

Noel et al. reported that known *Salmonella* pathogenicity genes such as *hilA*, *flhDC*, *fliF* and pSLT virulence plasmid encoded genes did not significantly contribute to survival fitness in the fruit (83). *hilA* is major gene regulator of invasion genes present on *Salmonella* pathogenicity island 1 (SPI-1). The *hilA* mutant deletion resulted in higher competitive fitness of the pathogen in tomatoes. Deletion of the pSLT virulence plasmid demonstrated its noninvolvement during survival in red ripe tomatoes. Flagellated, non-motile mutants of *Salmonella* produced by the disruption of the *motA* gene were as competitive as the wild type. The non-flagellated mutant (*fliF* deletion) was also as competitive as the wild type indicating that the presence of flagellum might not positively affect competitive fitness of *Salmonella* inside fruit tissue (83, 107). Studies have shown that *Salmonella* flagellar genes were required for attachment to lettuce leaves and might also be involved in chemotaxis towards plant exudates (67). These studies highlight the fact that different genetic machinery and strategies might be involved, depending upon the produce type, tissue and environment *Salmonella* serotypes might be exposed to.

Beauchat and Mann studied the survival of acid adapted and unadapted *Salmonella* in and on raw tomatoes. They found that the exposure of *Salmonella* to acidic conditions (pH 4.75) before inoculation did not affect their survival and growth in the stem scar and pulp tissue of Round and Roma tomatoes. It was also observed from the study that the population of

Salmonella increased significantly in the stem scar and pulp tissue over time. Higher populations were attained at 21°C than at 12° C. The pH values of the Round and Roma tomatoes in the pulp tissue were approximately 4.16 and 4.28 respectively. It was also observed that there was an increase in pH as the fruit matured over time (16). These studies indicate that *Salmonella* can adapt to the acidic environment inside fruit and can grow in internal tissues. Tomato fruit pulp and stem scars, when infected with *Salmonella* could result in an increase in population of the pathogen. Understanding patterns of entry into fruit is important for the development of effective pre-harvest and post-harvest control methods.

Flower as a route of *Salmonella* entry

Salmonella serotypes can survive and replicate not only on the phyllosphere or rhizosphere and on the surface of fruit but also in internal tomato tissues (49, 100). The acidic environment of the fruit pulp does not provide an effective barrier to the pathogen (16). Surface disinfection of the fruit does not ensure a safe product for the consumer as internalization of the pathogen has been demonstrated. Various studies have demonstrated pathogen entry into internal fruit tissue (49, 50, 118).

Zhuang et al. conducted a study on the fate of *Salmonella* Montevideo strain G4639 on the surface and stem-core tissues of mature green tomatoes (Table 2.3). The handling practices and conditions from the packing facility to the retail outlet were simulated to understand factors that affected survival, growth and internalization of the pathogen into fruit tissue. The effect of dip suspension temperature in promoting internalization into the stem core tissue was investigated. They found that the population of *Salmonella* increased significantly on the intact surface of the fruit when stored at 20°C and 30°C. A significant number of *S. Montevideo* cells were also internalized into the core tissue when tomatoes at a temperature of 25°C were

immersed into a suspension of 10°C. The populations of *S. Montevideo* present in the core tissue underwent a significant increase when stored at a temperature of 20°C over time. Chlorine treatments were less lethal to *S. Montevideo* when present in core tissue than on fruit surface (118).

Tomato plants were grown hydroponically in Hoagland solution (hydroponic growth nutrient solution) containing a 5 strain *Salmonella* cocktail. *Salmonella* was detected on hypocotyls, cotyledons, stems and leaves of plants regardless of the presence or absence of roots. None of the tissues were surface sterilized before analysis, indicating a possible uptake by capillary action. The study was one of the first to indicate a possible endophytic relationship of *Salmonella* serotypes with tomato plants (Table 2.3) (51).

Studies have shown that *Salmonella* can be transferred to tomato plant tissue (phyllosphere and rhizoplane) from contaminated soil (Table 2.3). Barak and Liang showed that *Salmonella* can survive in fallow soil for a period of six weeks and be capable of attachment and contamination. The population of *Salmonella* in the phyllosphere was stable for a period of 5 weeks. The pathogen was introduced to the soil through irrigation water. They showed that plant debris was also capable of causing contamination of the phyllosphere and rhizoplane with *Salmonella*. Tomato seeds sown in soil 24 hours after the soil had been fortified with contaminated plant debris resulted in crop that was positive for *Salmonella* on its surfaces (12). Miles et al. investigated the potential of *Salmonella* internalization into fruit from contaminated irrigation water and seed stock. The plants grown in a greenhouse received progressively higher levels of *S. Montevideo* contaminated water. The fruits, roots, stems and leaves were sampled. The study revealed that none of the fruit sampled was positive for *Salmonella*. The same result was observed for fruit produced from the contaminated seed stock. The stem and leaf sections

tested for *S. Montevideo* were also negative. It was observed that five of the root samples were positive for the presence of *Salmonella* (78). In the study by Miles et al., surface sterilization was performed on tomato fruit before *Salmonella* detection. Fruits were tested for internalization and not for presence of the pathogen on the surface.

Guo et al. demonstrated that upon contact of tomato fruit with *Salmonella* contaminated soil and storage at ambient temperature (Table 2.3); *Salmonella* could enter the stem scar and internal surface of the fruit (50). *Salmonella* internalization into tomato fruit has been achieved through techniques such as brushing of blossoms with inoculum, vacuum cycles and stem injection (100). The presence of wild animals and water runoff might have resulted in the presence of *Salmonella* on tomatoes in a hydroponic tomato facility. *Salmonella* was also found in water puddles, soil, shoes and feces of local wild and farm animals in the same facility. It should be noted that tomatoes were tested for surface contamination and that internal or core tissues were not evaluated for the presence of *Salmonella* serotypes after disinfection (86).

From these studies it can be observed that presence of *Salmonella* serotypes in the production facility where tomato plants are being grown can lead to contamination of the plant and fruit surfaces. Internal core might also get contaminated with *Salmonella* spp. if the fruit comes in contact with contaminated soil or if there is a temperature difference between the fruit and the dip solution as water can be absorbed when the temperature of the fruit is higher than the water it is being immersed into (50, 118). Another method of contamination that has led to internalization in studies is the introduction of inoculum into blossom (Table 2.3) (49, 100).

Guo et al. studied the fate of *Salmonella* in tomato plants during the flowering stage and stages of fruit formation and development (Table 2.3). A five *Salmonella* serotype cocktail was introduced before and after fruit set by injecting the stems and brushing the flower with

inoculum. Fruits were tested for *Salmonella* by testing the wash water and internal tissues such as the stemscar and pulp. The fruits were surface sterilized with 70% ethanol before testing. Two of the 8 tomatoes formed after flower inoculations were positive for the presence of *Salmonella*. Shi et al. studied the persistence of *Salmonella* serovars in pre and post-harvest tomatoes. Internalization was achieved by inoculating 100 µl of *Salmonella* suspension into flowers, following which fruits were allowed to develop for 6 to 7 weeks and tested after harvesting during early breaker stage. Both the surface and the internal tissue of fruit produced from inoculated flowers were positive for the presence of *Salmonella*. It should be noted that most serotypes were isolated after enrichment procedures(100). This could indicate that *Salmonella* serotypes can gain entry into the fruit through the flower and might require an enrichment procedure for detection.

The biocontrol potential of a combination of *Enterobacter asburiae* JX1 and a cocktail of 5 lytic bacteriophages was tested against *S. Javiana* within the rhizosphere of tomato plants and pre- and post-harvest tomatoes. *Salmonella* was inoculated with and without the biocontrol agents to facilitate entry on fruit surface and in fruit tissue. *S. Javiana* was recovered from 92% of surface rinses and was present internally in 43% of the fruit in which the flowers had been inoculated with the pathogen alone (117).

Blossom to fruit formation takes 20-30 days in tomatoes (Table 2.2) (63) .Blossoms might serve as a point of entry into fruit for other pathogens such as *Erwinia amylovora* in both pears and apples. Nectar in the blossoms could serve as a nutrition source to plant pathogens. It has also been observed that rain could aid in transfer of plant pathogens from the stigma to the nectarine region leading to infection. *Pseudomonas syringae* is another plant pathogen capable of establishing itself inside the flower and producing infection of flower tissue (57).

It can be observed from these studies that *Salmonella* transmission on to fruit surface and internal tissue could occur through contamination of the flower. Flowers have been known to serve as a source of penetration, point of infection and a niche for plant pathogens (57). It is important to determine if transmission of *Salmonella* could occur naturally to tomato blossoms from environmental reservoirs. Soil particles, water droplets, bird excreta, poultry and farm debris could all be transmitted by wind currents and have the potential to serve as sources of *Salmonella* on the field (73).

Table 2.2. Timeline for seed to fruit production of tomato crop (63)

	Stage of Development	Time period (Days)
0	Leaf Germination	25-35
1	Vegetative	20-25
2	Flowering	20-30
3	Fruit Formation	20-30
4	Fruit Ripening	15-20
Total time		100-140

Table 2.3. List of studies demonstrating *Salmonella* inoculation onto tomato fruit/ plant/ farm environment and effect of inoculation technique on presence of pathogen inside surface sterilized fruit tissue

<i>Salmonella</i> serotype used	Method of introduction	Tissue present	Presence inside tissue of surface sterilized fruit	Reference
Montevideo G4639	Tomatoes tempered at 25 ° C dipped in suspension at 10 °	Core tissue of tomato	Yes	Zhuang et al. 1995(118)

	C			
Montevideo Michigan Poona Hartford Enteritidis	Stem injection Flower brushing	43% from stem injection 2/8 for flower brushing	Yes Yes	Guo et al. 2001(49)
Montevideo Michigan Poona Hartford Enteritidis	Inoculated soil contact	Skin surface Subsurface Stem Scar	Yes	Guo et al. 2002(50)
Montevideo Michigan Poona Hartford Enteritidis	Hydroponic growth	Hypocotyl- cotyledon Stem Leaf	Not tested	Guo et al. 2002(50, 51)
Javiana 5913 Javiana 6027 Newport Montevideo Hadar Enteritidis Typhimurium Dublin Senftenberg Infantis	Flower inoculation Vacuum mediated internalization	Internal fruit tissue Tissue surface (washes)	Yes	Shi et al. 2007(100)
Montevideo Newport Strains of the F serogroup	Wild animals Water runoff in hydroponic facility	Isolated from tomatoes	Not tested	Leopoldo et al. 2008(86)
Baildon strain 05x-02123 Enteritidis strain 99A-23	Soil Crop debris Plant pathogen <i>X.campestris</i>	Phyllosphere Rhizoplane	Not Tested	Barak et al. 2008(12)
Javiana	Flower inoculation Rhizosphere/ soil inoculations Vacuum release	Internal core tissue/ calyx Fruit surface Root	Yes	Jianxiong et al. 2009(117)

	cycles			
Montevideo	Contaminated irrigation water and seed stock	Root	Not present in fruit	Miles et al. 2009(78)
Baildon	Soil	Fruit surface	Not Tested	Barak et al. 2011 (11)
Cubana	Water	Pedicel		
Enteritidis	Fruit	Calyx		
Havana	Leaf inoculation	Trichomes		
Mbandaka				
Newport				
Poona				
Schwarzengrud				

Environmental Factors

Animals are the primary hosts of *Salmonella* and the enteric pathogen possesses genes to invade, survive host cells and resist immune defense mechanisms(113). *Salmonella* also has genes that confer fitness in non-host environments. There are more than a 1000 genes in *Salmonella* with unknown functions. Some of these genes of unknown functions could be involved in aiding the pathogen's survival in soil and water. The alternative sigma factor RpoS is expressed both in *E. coli* and *Salmonella* during exposure to sea water. The alternative sigma factor is activated during stress responses such as those encountered in a non-host environment. *E. coli* with the RpoS deleted had a 1000 fold decrease in culturable cells while *Salmonella* cells had only a 10 fold decrease after both organisms were exposed to seawater for 8 days. This indicates that *Salmonella* might be more adapted to survival in adverse environmental conditions and might have additional genetic machinery aiding its survival in the environment (35, 48). Several species of reptiles are also known reservoirs of Salmonellae. It is also known that birds and insects might serve as vectors of *Salmonella* (81, 112). The majority of cases caused by *Salmonella* are foodborne and might result from undercooked beef, chicken, eggs and produce

such as a fresh fruits and vegetables (53). This indicates that a cyclic lifestyle could exist as the pathogen transitions from one host to another with intermittent environmental survival. From the apparent diversity of hosts such as animals, birds, reptiles and survival in diverse niches such as a soil, water and plant tissues it can be observed that the lack of specific host adaptations has enabled colonization of a wide variety of hosts possible (116).

In the United states, animal grazing is substantial enough to affect water quality, hence farms with large animal population could increase the risk of irrigation water contamination (69). The application of soil with manure produced by animals that are pathogen carriers could introduce foodborne pathogens in the farm environment (59). Several studies have demonstrated the persistence of *Salmonella* in the farm environment. The pathogen was detected in dairy farms, piggeries and slaughterhouse facilities both before and after slaughter (9, 58, 79). In a Danish piggery, it was observed that *Salmonella* Typhimurium DT12 clone was persistent in the herd environment. The pathogen was isolated for up to 14 days from soil where contaminated slurry had been disposed. The single clone was also isolated from the animals, feed and their environment over a period of 2 years indicating persistence of the strain (9). *Salmonella* persistence for over a year was also observed in a poultry house indicating that certain environments might be contaminated for extended periods of time by the pathogen (33). Contamination of plant surfaces with *Salmonella* is also possible when manure (containing pathogen) amended soil is used to grow plants (12, 116). Thus establishment of persistence in fields, water sources, farms and poultry houses could also increase the risk of produce contamination when grown in proximity to potential sources of *Salmonella*.

While animals, birds, insects and reptiles can host Salmonellae and aid in their spread, the survival of the pathogen on abiotic surfaces must be understood to predict transfer to produce

on the fields. The field environment is a dynamic one, with interplay of physical, chemical and biological factors. An understanding of these factors along with strategies for attachment and survival of *Salmonella* spp. could help in better determining risk factors and negating them. Salmonellae have been isolated from water sources and water might aid in transmission of the pathogen on to produce (25, 47). *Salmonella* also exhibit resistance to environmental fluctuations that might occur in marine environments resulting in improved survival in hydrological niches. It was observed that *Salmonella* outlived both *Staphylococcus aureus* and *Vibrio cholera* in ground water and heavily eutrophied river water indicating its versatility in survival (23). The dissemination of *Salmonella* into marine environments could aid in rapid dispersal of the pathogen. *Salmonella* has also been known to survive in a septic system for a 10 to 15 day period, and thus could enter fresh or agricultural water due to storm run-off or accidental leaks of sewage or septic tanks (88). Greene et al. conducted traceback studies to investigate two *Salmonella* Newport outbreaks, caused in 2002 and 2005 by the same PFGE pattern. Environmental sampling revealed that the rare strain responsible for the outbreaks was isolated from irrigation pond water near the farms two years apart. This indicates persistence of the strain and a long term contamination of the produce on the field. The pond water did not come in direct contact with the plant and entered into the soil bed though one of the growers had used the pond water for pesticide application on the plant. It was observed during the study that the cross connections in the water supply could have been contaminated and also that the pond water could have seeped through the soil and have been mixed with well water that was used to recharge the pond. Geese and turtles were also observed near the pond (47). Environmental investigations in fields during traceback after a multi-state tomato associated outbreak caused by *Salmonella* Braenderup revealed that there were animal, bird and reptile reservoirs of *Salmonella*

near the field. The drainage ditch water and animal feces samples near the tomato fields were also positive for multiple serotypes of *Salmonella* (4).

Rain runoff, underground water, surface water currents can all aid in the dissemination of *Salmonella* in agricultural soil and sediment (23). Under certain conditions, percolation of contaminated water through soil filters out bacteria that can persist in the field environment. *Salmonella* has been isolated from soils in agricultural areas and adhesion to soil particles might be related to cell surface hydrophobicity (104). Soil and sediment could act a reservoir of organic molecules for bacterial nutrition or a substratum for attachment and hence serve as a niche for pathogenic bacteria (23, 108, 116). The potential of wind currents in disseminating bacteria attached to soil and dust to plant surfaces requires to be explored. Understanding attachment strategies to soil particles could help understand how *Salmonella* spp. persist on the field and are transferred from host to host. Hendricks observed that there was an increased recovery rate of *Salmonella* from bottom sediments than surface water when tested for the presence of the pathogen. The adhesive property of the pathogen could have resulted in better recovery since dissemination is more rapid on the surface (55).

Common serotypes such as *S. Newport* and uncommon serotypes such as *S. Braenderup* have both been associated with tomatoes and traceback investigations have indicated that the pathogen may be persistent on the fields (7, 52). Understanding environmental survival strategies of serotypes associated with *Salmonella*-tomato outbreaks could help determine routes of transmission as these still remain unknown. Survival of pathogens in soil is considered a risk factor and the suggested time gap between manure application and growth of crop varies between a 120 days and a year (36). It is important to understand the survival of pathogens in soil to develop strategies to reduce produce contamination risk.

Environmental vectors

Several tomato outbreaks are indicative that contamination of fruit could occur (12). *Salmonella* serotypes have been successful in colonizing different plant tissues Barak and Liang demonstrated that *S. enterica* contaminated soil could lead to contamination of tomato plant phyllosphere. Also the pathogen could survive in fallow soil for six weeks with the ability to contaminate tomato plants (12). Previous research has shown that tomato fruit can be contaminated through root inoculation of the pathogen during hydroponic cultivation of the plant and also through inoculation of tomato blossoms (49, 51). It is known that *Salmonella* serotypes can survive on tomato plant tissue and *in planta* but the actual source of the pathogen remains ambiguous (12, 17).

Irrigation sources, soil, manure, insect and avian sources have been suspected sources of *Salmonella* spp. in produce related outbreaks (17). The specific route of *Salmonella* transmission in produce deserves deeper exploration to determine on-field risk factors and to develop practices that negate them. Animal production areas are considered high risk for produce contamination because *Salmonella* serotypes can survive for extended periods of time in transition between animals, excreta, soil and plants (61). Soil or manure containing *Salmonella* spp. might be transported by water and lead to transfer of bacteria on to plant tissue. Bacteria have been known to attach to soil particles because of cell charge or cell hydrophobicity. Huysman and Verstraete demonstrated that strains that were hydrophobic had 2-3 times slower percolation through soil columns (60). Biofilms might also play an important role to pathogen attachment to soil and transfer. Increased retention of *E. coli* has been observed due to the formation of biofilm (2, 19). A study by Salvucci et al. tested the relationship between biofilm formation and thin aggregative fimbriae (tafi) production and the transport of environmental

Salmonella spp. *Salmonella* spp. showed lesser retention even though the ability to produce biofilm was similar to *E. coli*. This indicates higher risk of infiltration through soil and thereby contaminating ground water and possibly the eventual contamination of produce (61).

While soil and water have been known to harbor *Salmonella* serotypes, airborne transmission of *Salmonella* spp. has also been investigated. Barker and Bloomfield conducted toilet seeding experiments designed to mimic conditions of acute diarrhea. Their study resulted in isolation of *Salmonella* from air samples immediately post flushing indicating possible airborne transmission (13). Intranasal inoculation of pigs with *Salmonella* Typhimurium resulted in appearance of the pathogen in the gut indicating that airborne transmission might be a potential alternate infection route for pigs (38).

Different environments could give rise to aerosols harboring bacteria. Animal and poultry bird confinement have been known to increase overall microbial load in the immediate environment. Agricultural practices such as spray irrigation of waste water and land application of biosolids or manure could lead to formation and dispersal of bioaerosols (34, 80). Biological materials in air could include particulate linked bacteria and does not necessarily occur as individual particles. Hence bioaerosols generated indoor could get dispersed outdoor depending on the particulate matter it is associated with and the environmental factors present (71). Various factors such as environmental conditions, nature of attached particulate and the persistence of the serotype might contribute to the continued survival of *Salmonella* in source material such as dust or litter.

Chinivasagam et al. assessed the levels of *Salmonella* and *Campylobacter* in aerosols present within and outside poultry sheds. It was found that the levels of bacteria in the air are

related to the level of the same bacteria in the litter. The *Salmonella* serotypes isolated in litter during the study were generally also isolated from aerosols and dust (26).

Lues et al. studied the microbial composition of a high-throughput chicken slaughtering facility over a four month period. *Salmonella* serotypes were detected in the facilities. Highest microbial counts were found in the receiving killing and defeathering areas. A strong correlation was found between *Salmonella* spp. presence and airborne particulates. *Salmonella* spp. was rarely isolated from other processing areas indicating that the organism might be introduced into the facility by dust spread by the birds. Dust was the only environmental factor to have an influence of significance on the dispersal of *Salmonella* spp (73).

These studies indicate that *Salmonella* serotypes can associate themselves with particulate matter and can be dispersed from their source. Water, soil, manure are suspected as sources of contamination on the field. It has been shown that these can be aerosolized and lead to pathogen spread (17, 80). The potential of airborne or dust associated *Salmonella* serotypes as a contamination threat in agricultural environments requires to be evaluated to aid in better risk evaluation. Real time imaging or *inplanta* detection of pathogen contamination of produce might shed light into the phenomenon of *Salmonella* internalization.

***Salmonella* biofilms**

A biofilm can be defined as a surface associated assemblage of bacteria enclosed in an self-produced extracellular matrix (96). Biofilms can contain single or multiple species of bacteria forming a single layer or three dimensional structures (102). They offer protection, from environmental stress, disinfectants and antimicrobials, to bacteria and contribute to their persistence. Several studies have shown that *Salmonella* spp. are capable of biofilm production on both biotic and abiotic surfaces (103). Biofilm formation by *Salmonella* on abiotic surfaces

could aid in its transmission to new hosts, since biofilms offer protection and improve persistence in non-host environments (116).

Stepanovic et al. tested 122 *Salmonella* serotypes isolated from food, animals and humans for their biofilm production capabilities and found that all the serotypes produced biofilm in plastic microtiter plates. *Salmonella* spp. produced more biofilm in comparison to *Listeria monocytogenes*. It was also observed that *Salmonella* spp. produced increased biofilm in nutrient poor medium in comparison to *L. monocytogenes* (105). Similar biofilm formation patterns were observed by Speranza et al, who studied the effects of nutritional and environmental conditions on *Salmonella* spp. biofilm formation. The strains changed from planktonic to surface attached mode found in biofilms, in nutritionally limited environments and suboptimal temperatures (102). This might indicate that *Salmonella* biofilms might facilitate survival in the environment where conditions might be sub-optimal.

White et al. indicated that the rdar morphology, could play a role in transmission between hosts (115). The rdar (red dry and rough) morphotype is a multicellular behavior resulting from growth on congo red agar and is linked with the expression of curli and fimbriae (103). *S. Typhimurium* cells in rdar colonies survived desiccation better than mutants deficient in fimbriae and cellulose production (115) and had better survival rates.

While biofilms aid in persistence salmonellae in the environment, they might also aid in attachment to plant tissue and internalization (103). It is known that *Salmonella* spp. follow a cyclic lifestyle that transitions between the host and environment. Biofilm formation has been known to protect the organism from environmental stresses and challenges (116). Many studies have shown that *Salmonella* serotypes attach to plant tissue using biofilms although plants until recently were not considered as traditional *Salmonella* hosts (103). Lapidot et al. studied the

adhesion and persistence of *Salmonella* Typhimurium to parsley leaves and the effect of chlorine based disinfection. It was observed that biofilm formation capability improved resistance to chlorination (68). The authors also observed that other mechanisms such as tissue penetration or interaction with pre-existing biofilms might aid in resistance to chlorine disinfection (6). Gandhi et al. demonstrated that *S. Stanley* was present at a depth of 12 μm within intact alfalfa sprout tissue indicating the possibility of internalization and survival of the pathogen inside plant tissue (42).

Biofilm production can protect *Salmonellae* from hostile conditions such as nutrition depletion and result in enhanced recovery from challenging environments. Also, the production of biofilm can enhance persistence on the phyllosphere by aiding attachment and protecting the pathogen from disinfection techniques. While biofilms have been studied on surfaces common in the food industry such as stainless steel, cement and plastic, their production has also been documented on biotic substances such as gallstones. It was demonstrated *S. Typhi* biofilm formation could be induced *in vitro* on gallstones. Prouty et al. also compared differences in bacterial biofilm formation on gallstones and pebbles under specific conditions and observed tightly compacted bacteria on the pebble surface with very little exopolysaccharide (93, 103).

Biofilm formation can be measured by culture based methods where sessile cells are dislodged by vortexing or sonication. Alternate techniques have been developed for quantitation of biofilms such as crystal violet staining and Syto9 staining (30). Stains like crystal violet can be used to quantitate cells in biofilms. Crystal violet (CV) staining was first described by Christensen et al. (27) and has since been modified to increase its accuracy and to allow biofilm biomass quantification. CV binds to negatively charged surface molecules and polysaccharides in the extracellular matrix (70, 90). Dye extraction was performed by Prouty et al. to determine

the amount of dye retained by bacterial cells attached to glass slides (94). The potential of this technique to determine the amount of bacteria attached to particles such as sand or dust needs to be explored. Studies on the association of *Salmonella* spp. with potential vectors such as dust and soil could help us better understand transfer of the pathogen from different hosts and possibly improve control over transmission.

Biophotonic imaging *in planta*

Culture based or molecular methods could help in enumeration of bacterial populations, detection of pathogen in plant tissues and study pathogen translocation patterns through different tissues. Certain limitations occur with use of these techniques. These methods are often invasive as the plant tissue requires to be excised and cannot be studied in real time. Excising plant tissue for study has the potential to dislodge cells from the surface to internal tissue. It was observed that cutting stemscar inoculated fruit with a sterile knife could transfer the pathogen to internal tissue and that the contaminated knife could also aid in cross-inoculation of sterile fruit (72). Excising tissue could actually lead to transfer of pathogen from surface to internal tissue due to mechanical action of the scalpel or knife. Also pathogens might not be culturable due to environmental stresses and counts might not represent actual populations present (116). The use of biophotonic imaging could overcome limitations of culture and certain molecular detection methods and be useful in studying whole plant or fruit and understanding pathogen interaction with host in real time.

Biophotonic imaging is a non-invasive, non-toxic method of optical imaging using visible light that is emitted during bioluminescence or through excitation, during fluorescence. Sensitive photon detectors such as cooled or intensified Charge Coupled Device (CCD) cameras can be used to detect light emitted through tissues. Light tight specimen chambers are used during

imaging to reduce background noise as the light emitted is often weak (3). Light emission through bioluminescence is the result of a chemiluminescent reaction between a substrate molecule that emits light known as a luciferin and a category of enzymes known as luciferases. Luciferin is found in luminescent bacteria such as *Photobacterium phosphoreum*, *Vibrio harveyi* and the terrestrial bacterium *Photorhabdus (Xenorhabdus) luminescens*. The luciferin in bacteria is a reduced riboflavin phosphate (FMNH₂) that is oxidized by a luciferase in association with a long-chain aldehyde and an oxygen molecule resulting in the emission of photons or light (48). The *lux A* and *B* genes code for luciferases while the *lux C*, *D* and *E* genes code for proteins that divert tetradecanoic acid away from the fatty acid biosynthesis pathway into the long chain fatty aldehyde substrate for the luminescent reaction (48, 76). Engebrecht et al. showed that the *lux CDABE* operon from *Vibrio fischeri* could be vectored into and expressed in *E.coli* without the addition of a substrate because of the presence of the complete operon (37). The *lux CDABE* operon of the bioluminescent bacterium *Photorhabdus luminescens* is an excellent reporter operon that encodes for luciferase and substrate production, hence not requiring external addition of substrate. Its thermostability and optimum activity at mammalian body temperatures makes it an effective tool for *in vivo* studies (99).

Bioluminescence has many research applications targeted toward understanding bacterial pathogenesis, monitoring gene expression non-invasively, monitoring hygiene and food safety research. ATP swabbing using firefly luciferase is common place and has been used to determine hygiene and sanitation standards of surfaces. Phages transformed with luciferase gene and has been used for the detection of specific pathogens in food and environmental samples (48). In 1995, Contag et al. demonstrated that it was possible to monitor disease processes in living animals using bioluminescence (31, 41). Since then bioluminescence has been used to

study pathogen host relationships in both animals and plants. Warriner et al. studied establishment of *E.coli* and *Salmonella* Montevideo on and within mung beans. The study showed that *E.coli* and *Salmonella* present on seeds could be internalized into sprouts and cannot be removed by biocidal washing procedures (114). Chen and Griffiths used *lux* genes in *Salmonella* Enteritidis as a reporter to study the effects of pH and temperature on survival and recovery of cells. They also reported that plasmid mediated luminescence was superior to recombinant cultures in light emission. This could have resulted from the high copy number of the plasmid (24). Karsi et al. developed 12 bioluminescent strains in *Salmonella* isolated from a chicken broiler continuum for real time monitoring in food products. They were able to develop a chicken skin attachment model using these strains (65).

Bioluminescent tagging of bacterial pathogens can be done by the integration of the operon into the chromosome or by the use of plasmids. Both these techniques have advantages. Integrating the *lux CDABE* operon into the chromosome reduces the amount of light produced and requires higher number of cells for detection. Transforming the plasmid through electrotransformation or conjugation could result in more light production due to the high copy number of the plasmid (24). Often plasmids are tagged with antibiotic resistance genes and luminescence can be lost when antibiotic pressure is lost in the environment (41). It was reported by Bautista et al. that only one of the seven *Salmonella* isolates from a poultry facility were successfully transformed with the *lux* plasmid in their study to determine the long term effects of lactic acid rinse on pathogen viability and recovery (14).

The development of bioluminescent strains of *Salmonella* from serotypes associated with tomato related outbreaks is important. Tomato outbreak associated bioluminescent *Salmonella* serotypes may help shed light on survival strategies and adaptations of the pathogens in real

time. Quantitative models of *Salmonella* attachment to different plant tissue and the effect of antimicrobials in reducing pathogen load can be determined in real time. Internalization patterns of *Salmonella* into fruit could be determined using imaging over time. Bioluminescent *Salmonella* serotypes can be used as a tool to study the potential of vectors in such as dust, plant debris, soil and water in transferring the pathogen, giving us a better idea of on-field dynamics.

To develop these tools, several factors need to be explored. These include methods to improve electro-transformation efficiency while transferring plasmids from *E.coli* into wild-type outbreak associated serotypes of *Salmonella*. van der Rest et al. found that the efficiency of electrotransformation with xenogeneic DNA is severely limited by the restriction system of the recipient. They demonstrated that a heat shock treatment could improve electro-transformation efficiency during transfer of xenogeneic DNA (foreign DNA) into *Corynebacterium glutamicum* (110). The potential of heat-shock treatment in improving transformation-efficiency in tomato-outbreak associated *Salmonella* serotypes needs to be explored as difficulty in electrotransformation has been observed. Plasmid stability without antibiotic selective pressure needs to be determined in outbreak associated serotypes. Differences could also occur in expression of the *lux* operon between different serotypes of *Salmonella* and hence choosing the right strain would help develop a robust research tool.

Biophotonic imaging can help develop more efficient research models to understand *Salmonella* interaction with tomato plants and to study factors that might lead to fruit contamination on the field. Photon emitting *Salmonella* serotypes that have been previously associated with tomato outbreaks could serve as important tools. To develop an efficient tool it is important to determine plasmid stability and developing techniques to overcome barriers associated with electrotransformation of xenogeneic DNA

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CHAPTER 3. SURVIVAL OF TOMATO OUTBREAK ASSOCIATED *SALMONELLA* SEROTYPES IN SOIL AND WATER AND THE ROLE OF BIOFILMS IN ABIOTIC SURFACE ATTACHMENT

Abstract

Presence of *Salmonella* in the field environment might increase risk of contact with produce resulting in contamination. To understand survival of tomato outbreak associated *Salmonella* serotypes in soil and water, their populations were enumerated over a 40 day period. Differential and non-selective media were used for enumeration purpose to assess growth and to account for cell injury. Based on differences in populations of *Salmonella* enumerated through dual plating, it was observed that *Salmonella* serotypes underwent more cell injury during survival in soil than water. No significant differences in population patterns were observed between serotypes. The ability of tomato outbreak associated *Salmonella* serotypes to produce biofilms was also tested. Cells in the red dry and rough (rdar) colonies are more suited for survival in the environment during transmission between hosts. All serotypes were capable of producing the rdar morphotype. All *Salmonella* serotypes tested were capable of biofilm based strong attachment on glass coverslips and quartz particles. Differences in rate and quantity of attachment were observed based on material ($P < 0.0001$). Also different patterns of attachment and release of cells were demonstrated between serotypes. The results indicate that *Salmonella* serotypes associated with tomato outbreaks could be capable of biofilm formation on abiotic surfaces and survival in soil and water, Biofilm based attachment to sand particles might serve as a strategy for environmental survival and dispersal of *Salmonella*.

Introduction

It is estimated that there are 48 million foodborne illnesses, 128,000 hospitalizations and 3,000 deaths each year in the United States (20). Produce associated outbreaks have burgeoned over the last three decades and tomatoes have been frequently determined as a vehicle of Salmonellosis (12). Outbreaks caused by uncooked produce such as tomatoes can result in massive economic losses due to drop in sales and recalled product (12). According to the United States Department of Agriculture – Economic Research Service (USDA-ERS), productivity loss and medical expenses from *Salmonella* related infections amounted to 2.7 billion US dollars in 2010 (1).

Tomato associated salmonellosis have resulted both from common human illness causing serotypes such as *Salmonella enterica subsp. enterica* serotype Newport and rare serotypes such as *S. Baildon* and *S. Braenderup* (7, 8, 11). *Salmonella* spp. are intracellular animal pathogens, that can lead a cyclic lifestyle during host to host transition (25). Barack and Liang showed that *S. Baildon* strain 05x-02123 and *S. Enteritidis* strain 99A-23 could survive in fallow soil for a period of 6 weeks and be capable of attachment and contamination of tomato plants (3). Guo et al. demonstrated that upon contact of tomato fruit with *Salmonella* contaminated soil and storage at ambient temperature; *Salmonella* spp. could enter the stem scar and internal surface of the fruit (10). Inoculation of tomato blossoms with *Salmonella* spp. can also result in presence of pathogen in internal fruit tissue (9, 21), but vectors of *Salmonella* spp. resulting in on-field contamination of tomatoes still remain ambiguous.

Traceback investigations of tomato associated *Salmonella* outbreaks have revealed that multiple serotypes of *Salmonella* spp. could exist in packing houses, farm environment (8, 11). Presence of *Salmonella* serotypes in soil and water could result in repeated outbreaks. Pond

water persistence was observed during traceback investigations involving the same strain of *S. Newport* in 2002 and 2005 (8). Biofilms are the predominant mode of bacterial growth in nutrient challenged environments and their formation can provide *Salmonella* resistance to stress in challenging environments (22).

Salmonella spp. are capable of biofilm production on both biotic and abiotic surfaces (22). The production of biofilm can enhance persistence on phyllosphere by aiding attachment and protecting the pathogen from disinfection techniques (14). Prouty et al. compared differences in *Salmonella* biofilm formation on gallstones and granite pebbles under specific conditions and observed tightly compacted bacteria on the pebble surface with very little exopolysaccharide presence (19). While cell surface hydrophobicity could result in adhesion to soil particles (23), the role of biofilms in attachment of *Salmonella* spp. to soil particles needs to be explored. Hence understanding survival patterns of *Salmonella* spp. in soil and water and testing for biofilm production capabilities of tomato outbreak serotypes could shed light on persistence strategies of the pathogen.

In this study the survival patterns of outbreak associated *Salmonella* serotypes in soil and water over a 40 day period was determined. The biofilm forming potential of tomato outbreak associated serotypes was also determined on abiotic surfaces such glass cover slips and quartz dust using Crystal Violet staining of attached bacterial cells (6, 19). It was hypothesized that biofilm production could be used as a strategy for survival, attachment and even transport to produce on the field by *Salmonella* spp. The ability of outbreak associated serotypes to survive in soil and water and produce biofilms during the time usually taken for transition from blossom to fruit set could indicate that soil and water could serve not only as reservoirs but also as vectors of on-field contamination.

Materials and method

Bacterial strains

Stock cultures of *Salmonella enterica* subsp. *enterica* serovar Newport, Javiana, Montevideo, Anatum, Baildon and Braenderup were obtained from the culture collection at Virginia Polytechnic Institute and State University. Frozen stock cultures were thawed and a loopful from each stock culture was transferred to separate 25 ml of Brain Heart Infusion broth (BHIB; Becton, Dickinson and Co, Sparks, MD) and incubated for 24 hrs at 37°C. One loopful of culture was transferred thrice at intervals of 24 hrs into 25 ml of BHIB and incubated at 37°C for 24 hr in a shaking incubator to facilitate complete revival. A loopful of cells from the final transfer into BHIB was streaked onto xylose lysine Tergitol 4 (XLT4); (Becton, Dickinson and Co, Sparks, MD) for presumptive determination of typical colonies and on Tryptic soy agar (Becton, Dickinson and Co, Sparks, MD) for growth on non-selective medium. The plates were incubated for 24 hrs at 37°C. Representative, cultures from the TSA plate was confirmed biochemically using 20E API strips (bioMerieux, Hazelwood, MO) and latex agglutination assay (Oxoid). Upon confirmation, colonies were streaked on TSA slants and incubated for 24 hrs at 37°C, following which the slants were stored in the refrigerator at 4°C for inoculum preparation.

Soil and water preparation

Soil was obtained from a tomato farm in the eastern shore of Virginia. The sandy soil was passed through a No. 20 sieve (U.S.A Standard Testing Sieve) to obtain a uniform grain size. The soil was autoclaved (121°C for 12 min) to eliminate populations of existing microorganisms in the soil. Autoclaved soil was tested for the presence of sterility by performing an aerobic plate count. The soil sample was also screened for the presence of *Salmonella* spp. by plating on

XLT4 agar. Autoclaved deionized distilled water was used to study survival patterns of *Salmonella* serotypes in water.

Inoculum preparation

A loopful of each *Salmonella* serotype (*S. Newport*, *S. Javiana*, *S. Montevideo*, *S. Baildon*, *S. Braenderup* and *S. Anatum*) was used to inoculate 25 ml of Tryptic Soy Broth (TSB) each. The broth was incubated at 37°C for 24 hrs in a shaking incubator at 100 rpm to maximize *Salmonella* cell density. After incubation the culture was centrifuged at 1400 g for 7 min. The supernatant was decanted. The pellet was washed twice with sterile distilled deionized water to rid the culture of nutrient media. The pellet was re-suspended in 5 ml of sterile deionized distilled water.

Inoculation of soil and estimation of Salmonella population

Fifty gram portions of autoclaved soil were placed in sterile specimen collection containers (Fisher Scientific, Inc) for inoculation with each serotype. Soil was inoculated with 5 ml of each *Salmonella* serotype that had been suspended in sterile deionized distilled water. Inoculation of soil was performed in 10g increments to ensure even distribution of the *Salmonella* serotypes in soil. Ten gram layers of soil were inoculated with 1 ml culture in increments and mixed with a sterile stirring rod until each container held 50 g of soil. The containers were incubated at 30°C over a period of 40 days and 1 g of soil was sampled every 10 days from each container. Population of *Salmonella* spp. in soil was estimated through spread plating 100 µl of soil-peptone water suspension on XLT4 and TSAYE. The dual plating system was used to estimate the level of stress related cell damage, based on difference in populations,

Inoculation of water and estimation of Salmonella population

Forty five milliliter portions of sterile distilled deionized water was inoculated individually with 5 ml of each serotype suspension in a sterile sample collection container and incubated at 30°C throughout the duration of the experiment. The sample collection container was shaken vigorously to ensure even suspension of the bacterial cells in water. The containers were incubated at 25°C over a period of 40 days and 1 ml of the suspension was sampled every 10 days. Population of *Salmonella* spp. was estimated through spread plating spread plating on XLT4 and TSAYE. The dual plating system was used to estimate the level of stress related cell damage, based on difference in populations.

Curli and cellulose expression

Salmonella serotypes Anatum, Baildon, Braenderup, Montevideo, Newport, and Javiana were grown in TSB for 18 h at 37° C incubation. A loopful of inoculum of each serotype was streaked on separate Luria Bertani (LB) plates without salt supplemented with Congo Red (40 µg/ml) and Coomassie brilliant blue (20 µg/ml) (Congo red agar plates). Colony morphologies were assessed on C R plates after growth at 28° C for 48 h for individual colonies with red dry and rough morphotype which is indicative of curli and cellulose expression by serotypes tested.

Growth of biofilm on glass coverslips

Glass coverslips were placed in the bottom of a petri dish and covered with 30 ml of tryptic soy broth (TSB). Each individual serotype was inoculated into plates containing coverslips using 1:100 dilutions of overnight cultures to compare differences in attachment and biofilm formation between *Salmonella* serotypes. For controls, similar setup was used without inoculating the TSB. Plates were rocked slowly (30 rpm) overnight at 37°C. Every 24 h the

medium was removed; the cover slips were thoroughly washed two times with 1x phosphate-buffered saline (PBS). Enough cover slips were placed into the petri dishes so that one could be removed every 4 days for biofilm estimation for 14 days.

Growth of biofilm on quartz particles

Quartz particles were used as a surrogate for sand particles to facilitate Crystal Violet staining due to uniformity in color. Twenty gram of quartz (50-70 mesh, Sigma-Aldrich) was placed in a sterile petri dish containing 20 ml of TSB broth. Each individual serotype was inoculated into plates containing quartz particles using 1:100 dilutions of overnight cultures to compare differences in attachment and biofilm formation between *Salmonella* serotypes. For controls, similar setup was used without inoculating the TSB. The petri dishes were placed in a shaking incubator at 30 rpms and allowed to incubate overnight. Every 24 h the medium was removed; quartz particles were washed two times in 1x phosphate-buffered saline (PBS). Two hundred and fifty mg (approximate weight of a coverslip) of the quartz particles was removed every 4 days for biofilm estimation for a period of 14 days.

Crystal Violet staining of glass coverslips

CV staining of sessile cells was adapted from procedure used by Prouty et al. (19) . Cover slips were removed from the petri dish and washed with a continuous spray of 1x PBS until all broth and debris was washed off. They were then incubated at 60°C for 1 h to fix the cells. Following which 0.1% crystal violet (gentian violet in isopropanol-methanol-1x PBS [1:1:18]) was placed on each cover slip, and the cover slips were incubated for 15 min at room temperature. The slides were then washed thoroughly with 1x PBS until the PBS ran clear. The slides were then broken, put in Eppendorf tubes, and immersed in 33% acetic acid to extract the

dye. The optical density at 570 nm of the acetic acid solution was assessed to determine the amount of dye retained by the bacterial cells.

Crystal Violet staining of quartz particles

Quartz particles that were incubating in the petri dish with *Salmonella* culture were washed with a steady spray of 1x PBS until all broth and debris was washed off. Two hundred and fifty mg of the quartz particles were transferred to an eppendorf tube. These were then incubated at 60°C for 1 h to fix the cells. Following which 0.1% crystal violet (gentian violet in isopropanol-methanol-1x PBS [1:1:18]) was used to stain the particles. The quartz particles were then washed thoroughly with 1x PBS until the PBS ran clear. The particles were then immersed in 33% acetic acid to extract the dye. The optical density at 570 nm of the acetic acid solution was assessed to determine the amount of dye retained by the bacterial cells.

Statistical analysis

The experiment to determine survival patterns of *Salmonella* serotypes- Anatum, Baildon. Braenderup, Javiana, Newport, Montevideo in soil, water and studies on attachment to glass and quartz dust was a randomized blocked design factorial treatment arrangement, repeated measures with sampling, blocked on replication. Water and soil survival of *Salmonella* serotypes was analyzed using PROC GLM SAS 9.1 (SAS Institute Inc., Carey NC). The significant difference between the means was established by Least Significant Difference (LSD) Tests. Variables included for analysis were *Salmonella* serotype, vector and day. Attachment patterns of *Salmonella* serotypes to quartz dust and to glass cover slips was analyzed using GLM procedure. Comparison of Least Square Means (LSM) of O.D values was used to analyze cell

attachment on glass and quartz over time. A probability value of less than 0.05 indicated a statistically significant.

Results

Survival of Salmonella serovars in soil

Salmonella serovars were each individually inoculated into autoclaved soil, mixed evenly and stored in an incubator at 25°C for a period of 40 days. The soil was enumerated using XLT4 agar and TSAYE at 10 day intervals. All six serotypes used in this study had similar patterns of survival in autoclaved soil over a 40 day period at 25°C (P=0.72). The initial mean population for *Salmonella* serotypes on TSAYE was $\log 10.27 \pm 0.05$ CFU/g soil and 10.26 ± 0.1 log CFU/g soil on XLT4 (Table 3.1, 3.2). Different survival trends of *Salmonella* serotypes were observed on soil when enumerated on XLT4 and TSAYE, indicating stress or cell damage. A mean decrease to 6.60 ± 0.031 log CFU/g of soil by *Salmonella* serotypes was observed during TSAYE enumeration after 20 days of survival (Table 3.1). This was the lowest drop in population during 40 days. Stabilization from population loss at the 30 day period was observed from enumeration on TSAYE (Table 3.1). At the end of the 40 day period the mean population of *Salmonella* serotypes averaged 7.43 ± 0.26 log CFU/g on TSAYE and 5.15 ± 0.67 log CFU/g soil on XLT4 (Table 3.1, Table 3.2). XLT4 agar enumeration of *Salmonella* populations in soil did not indicate pattern of population recovery between day 20 and 30 (Table 3.2). Population recovery was observed between day 20 and 30 when enumeration was performed on TSAYE (Table 3.1). Populations of *Salmonella* serotypes changed in soil over time (P < .0001).

Survival of Salmonella serovars in water

Soil and water elicited different survival patterns in *Salmonella* serotypes, indicating that the medium in which the pathogen was present had an effect on population dynamics ($P < .0001$). No differences were observed between the six *Salmonella* serotypes in their survival patterns in water. Enumeration was performed on both TSAYE and XLT4. Initial mean populations of *Salmonella* serotypes during day 0 on TSAYE and XLT4 was 10.27 ± 0.06 log CFU/ml water and 10.27 ± 0.03 log CFU/ml water respectively (Tables 3.3, 3.4). *Salmonella* populations in water changed significantly with time. On day 10 there was a drop to 7.49 ± 0.26 log CFU/ml and 7.73 ± 0.15 log CFU/ml of water on TSAYE and XLT4 respectively (Tables 3.3 and 3.4). The mean population of *Salmonella* serotypes after 40 days was 7.71 ± 0.55 log CFU/ml water and 7.67 ± 0.67 log CFU/ml water on TSAYE and XLT4 respectively (Tables 3.3, 3.4). Hence a pattern of initial population drop at day 10 followed by stabilization of the population was observed, indicating survival was possible in water for a period of 40 days and that the level of cell injury incurred in water was lesser than that incurred in soil.

Biofilm formation on Congo Red agar

All six serotypes, Anatum, Baildon, Braenderup, Montevideo, Newport, Javiana produced red dry and rough (rdar) morphotype after 48 hours of incubation on Congo Red agar at 28°C indicating presence of curli fimbriae and cellulose production as components of their extracellular matrix.

Biofilm formation by Salmonella serovars on glass slides

Quantitation of biofilm density of six tomato outbreak associated *Salmonella* serotypes attached to glass surface was performed over a period of 14 days using modified CV method. All

the serotypes demonstrated an increase in the number of cells attached to the glass cover slip over time followed by a decrease in attached cells (Table 3.3). *S. Baildon*, *S. Braenderup* and *S. Newport* had maximum cell attachment to cover slips on day 10 while *S. Montevideo* and *S. Javiana* demonstrated maximum cell attachment to glass cover slips on day 6 (Table 3.3). *S. Anatum* showed progressive increase in cells attached over 14 days with the peak attachment on day 14 (Table 3.3). All the other serotypes demonstrated a drop in cells attached after a peak in cell attachment, indicating release into the environment. On glass surface, days 4, 10 and 14 were highly significant ($P < 0.0001$) for attached cell density indicating patterns of attachment and release of cells (Table 3.3).

Biofilm formation of Salmonella serovars on quartz particles

Quantitation of biofilm density of six tomato outbreak associated *Salmonella* serotypes attached to quartz particles was performed over a period of 14 days using modified CV method. We observed that maximum attachment of *S. Anatum*, *S. Braenderup*, *S. Newport*, and *S. Javiana* to quartz particles occurred on day 6 ($P < 0.0001$) (Table 3.4). There was a drop in cells attached to quartz particles after day 6 (Table 3.4). Maximum attachment to quartz particles occurred on day 2 followed by a drop in attached cells for *S. Baildon* (Table 3.4).

Discussion

Population patterns of tomato outbreak associated *Salmonella* serotypes in soil and water over a period of 40 days was studied through enumeration. Survival of *Salmonella* in soil and water for 40 days might indicate a contamination risk for tomato blossoms. Flower to fruit formation takes 20- 30 days in tomatoes (13) and studies have shown that inoculation of blossoms with *Salmonella* can result in the presence of the pathogen in fruit (9, 26). Hence

accidental or unintentional introduction of *Salmonella* into the environment during the flowering stage through animal feces, manure, contaminated irrigation water, biosolids and other sources could result in blossom contamination by *Salmonella* (4). Persistence of *S. Newport* in irrigation ponds has been associated with tomato recurrent outbreaks (8). Environmental traceback during other tomato associated *Salmonella* outbreaks have indicated the presence of *Salmonella* contamination sources in the field such as animal feces, birds and animals (7, 8, 11).

A dual plating technique was used to enumerate *Salmonella* populations over a 40 day period to account for cell injury over time. Dual plating technique has been used previously to account for cell injury in studies on *Listeria monocytogenes* survival and inactivation in brine (16, 17). TSAYE and XLT4 were used for enumeration and the difference in population on the selective and differential medium accounted for cell injury in *Salmonella* serotypes during survival in soil and water. The population of *Salmonella* changed with time ($P < 0.0001$), with different population trends being observed in soil and water.

From population enumeration on TSAYE for soil, it was determined that after an initial drop in population of *Salmonella* cells, there is stabilization or recovery of population levels. All soil samples used in this experiment were autoclaved and the presence of naturally occurring antimicrobials in soil was ruled out as similar population levels of *Salmonella* were observed in laboratory grade quartz sand over a 40 day period (unpublished data). The mean reduction in population of *Salmonella* serotypes in soil at the end of a 40 day period when enumerated on TSAYE was 2.84 log CFU/g soil. While the mean population at the 20 day period had dropped to 3.67 log CFU/g of soil indicating that there was a recovery over the next 20 days. This trend was not observed during simultaneous enumeration on XLT4 agar. These differences in growth patterns might indicate that populations of stressed or injured cells might not be accounted for

when differential agars such as XLT4 are used for enumeration. A similar pattern of steady decline in *Salmonella* spp. cell population has been demonstrated in previous study on survival of *S. Newport* in agricultural soils (27). The study used XLD agar for enumeration. Barak and Liang demonstrated that decline and stabilization of population levels could be observed during survival of *Salmonella* spp. in soil (3). Hence it should be noted that differential media might not account for stress and injury of *Salmonella* especially when the pathogen is present in a potentially stressful environment such as soil.

Less cell injury was observed during survival in water over a period of 40 days in *Salmonella* serotypes. A total mean reduction of 2.55 log CFU/ml and 2.94 log CFU/ml was observed on TSAYE and XLT4 respectively in water at the end of the 40 day period. Similar trends of population decline and recovery could be observed on both TSAYE and XLT4 agars indicating that less injury has been incurred by *Salmonella* serotypes in water over a period of 40 days. *Salmonella* have been known to survive in marine environments and overcome challenges such as salinity and temperature variations associated (25). Also long term persistence of the pathogen has been observed in irrigation ponds indicating that the pathogen might be better adapted for survival and transmission through water (8).

The ability of *Salmonella* to form biofilm on abiotic surfaces such as glass coverslips and quartz particles was studied through quantitation using a modified CV staining procedure. All six tomato associated *Salmonella* serotypes were tested for the production of red dry and rough (rdar) morphotype of Congo Red (CR) agar. The rdar morphotype production has been associated with environmental persistence (24) and is indicative of the production of curli fimbriae and cellulose by the pathogen. All six serotypes that were capable of rdar morphotype

production on CR agar were capable of biofilm production on quartz particles and glass coverslips.

The amount of biofilm material on cover slips and quartz particles was quantified using a modified CV staining method adapted from Prouty et al.(19). During the study, natural binding of dye to quartz particles was observed. Uninoculated quartz particles used as controls retained crystal violet and had a mean optical density (OD) of 0.47 ± 0.07 at 570 nm from the resealed dye, indicating binding .The OD readings of quartz samples with attached *Salmonella* cells were in some cases lower than the OD of the controls. CV binds to negatively charged surface molecules and polysaccharides in the extracellular matrix (15, 18). The presence of biofilm components on the quartz particles might have reduced dye interaction with particle surface. Quartz particles in controls might have retained more stain because of absence of competing material such as biofilm components.

From the attachment studies to glass slides and quartz particles, it was observed that attached cells can also be released into the medium over time, indicated by the drop in dye retention. In the natural environment, salmonellae attached to soil or dust have the potential to be dispersed by wind and water on the fields. Rain runoff, underground water, surface water currents can all aid in the dissemination of *Salmonella* in agricultural soil and sediment (5). *Salmonella* serotypes can survive in soil and water for extended periods (2, 27). *Salmonella* cells attached to soil through biofilms have the potential to be transported by wind and water activity on fields and be resistant to desiccation environmental stressors. *Salmonella* attached soil particulate dispersion might be an important risk factor for on-field contamination of produce.

The study indicates that it would be important to develop monitoring systems to prevent accidental and unintended introduction of *Salmonella* into soil especially during the blossom

stage of tomato crop. *Salmonella* is capable of survival in soil and water during the blossom stage. Biofilm formation might aid in the pathogen's attachment to abiotic surfaces and dispersal in the farm environment.

Table 3.1. Populations of *Salmonella* serovars in sterilized soil samples during 40 days of storage at 30° C as determined on TSAYE agar.

Day	<i>S. Anatum</i> CFU/g soil*	<i>S. Baildon</i> CFU/g soil	<i>S. Braenderup</i> CFU/g soil	<i>S. Montevideo</i> CFU/g soil	<i>S. Newport</i> CFU/g soil	<i>S. Javiana</i> CFU/g soil
0	10.18 ± 0.07	10.33 ± 0.04	10.31 ± 0.01	10.3 ± 0.02	10.25 ± 0.04	10.25 ± 0.06
10	7.54 ± 0.21	8.23 ± 0.3	8.68 ± 0.15	7.87 ± 0.52	7.7 ± 0.08	7.28 ± 0.01
20	6.75 ± 0.17	6.17 ± 0.48	6.38 ± 0.45	7.02 ± 0.37	6.77 ± 0.10	6.54 ± 0.09
30	7.98 ± 0.2	7.81 ± 0.12	7.82 ± 0.03	7.92 ± 0.15	7.82 ± 0.19	7.29 ± 0.86
40	7.14 ± 0.81	7.75 ± 0.17	7.25 ± 0.89	7.54 ± 0.11	7.23 ± 0.71	7.66 ± 0.22

*Soil obtained from a tomato production area on the Eastern Shore of Virginia; passed through a #20 sieve and autoclaved at 121°C for 12 min prior to use.

Table 3.2. Populations of *Salmonella* serovars in sterilized soil samples during 40 days of storage at 30° C as determined on XLT4 agar.

Day	<i>S. Anatum</i> CFU/g soil*	<i>S. Baildon</i> CFU/g soil	<i>S. Braenderup</i> CFU/g soil	<i>S. Montevideo</i> CFU/g soil	<i>S. Newport</i> CFU/g soil	<i>S. Javiana</i> CFU/g soil
0	10.43 ± 0.36	10.25 ± 0.06	10.24 ± 0.09	10.23 ± 0.05	10.26 ± 0.04	10.12 ± 0.10
10	7.95 ± 1.32	7.67 ± 0.18	8.62 ± 0.13	7.69 ± 0.04	7.02 ± 0.53	8.23 ± 0.30
20	6.82 ± 0.06	5.55 ± 0.18	6.56 ± 0.13	6.57 ± 0.16	6.40 ± 0.32	6.38 ± 0.02
30	6.25 ± 1.02	5.49 ± 0.60	5.84 ± 0.37	5.40 ± 0.53	5.36 ± 0.45	5.24 ± 0.63
40	5.15 ± 0.26	5.54 ± 0.13	5.91 ± 1.44	5.11 ± 0.56	5.28 ± 0.30	3.91 ± 1.35

*Soil obtained from a tomato production area on the Eastern Shore of Virginia; passed through a

#20 sieve and autoclaved at 121°C for 12 min prior to use.

Table 3.3. Populations of *Salmonella* serovars in sterilized distilled deionized water samples during 40 days of storage at 30° C as determined on TSAYE agar.

Day	<i>S. Anatum</i> CFU/ml water*	<i>S. Baildon</i> CFU/ml water	<i>S. Braenderup</i> CFU/ml water	<i>S. Montevideo</i> CFU/g water	<i>S. Newport</i> CFU/g water	<i>S. Javiana</i> CFU/g water
0	10.31 ± 0.02	10.28 ± 0.06	10.31 ± 0.02	10.24 ± 0.09	10.29 ± 0.05	10.15 ± 0.12
10	7.54 ± 0.03	7.61 ± 0.24	7.52 ± 0.13	7.77 ± 0.23	7.00 ± 0.35	7.47 ± 0.03
20	8.34 ± 0.01	8.31 ± 0.04	8.56 ± 0.28	8.72 ± 0.04	8.26 ± 0.21	8.53 ± 0.18
30	8.27 ± 0.05	8.16 ± 0.13	8.23 ± 0.10	8.16 ± 0.11	8.19 ± 0.04	8.33 ± 0.35
40	8.17 ± 0.04	6.67 ± 1.28	8.01 ± 0.13	7.59 ± 0.13	8.07 ± 0.13	7.72 ± 0.53

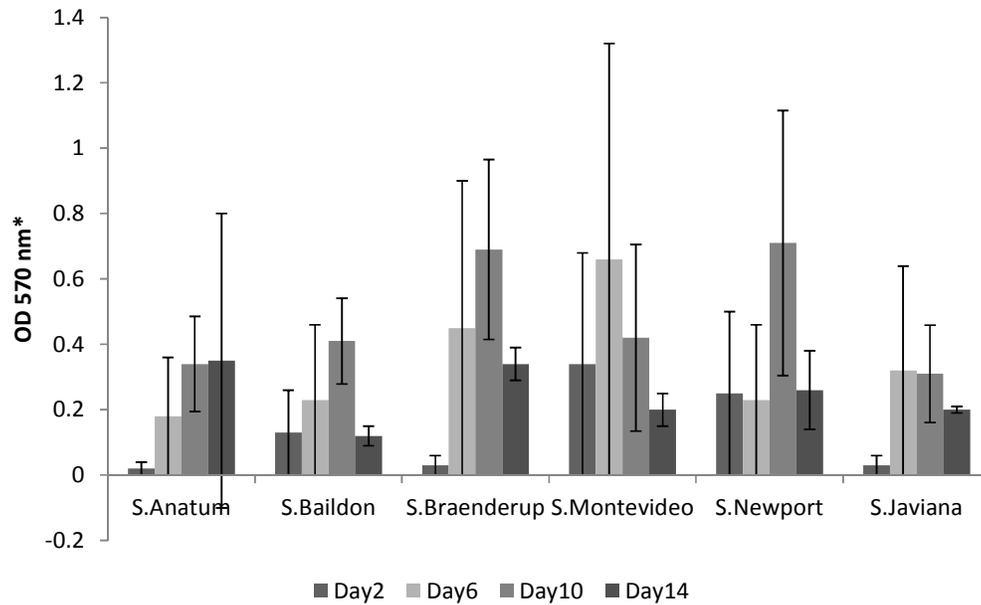
*Distilled deionized water was autoclaved at 121°C for 12 min prior to use.

Table 3.4. Populations of *Salmonella* serovars in sterilized distilled deionized water samples during 40 days of storage at 30° C as determined on XLT4 agar.

Day	<i>S. Anatum</i> CFU/ml water*	<i>S. Baildon</i> CFU/ml water	<i>S. Braenderup</i> CFU/ml water	<i>S. Montevideo</i> CFU/g water	<i>S. Newport</i> CFU/g water	<i>S. Javiana</i> CFU/g water
0	10.28 ± 0.04	10.29 ± 0.07	10.27 ± 0.05	10.28 ± 0.07	10.27 ± 0.07	10.2 ± 0.11
10	7.82 ± 0.36	7.74 ± 0.35	7.82 ± 0.46	7.59 ± 0.59	7.48 ± 0.12	7.88 ± 0.51
20	8.29 ± 0.04	8.35 ± 0.04	8.53 ± 0.28	8.82 ± 0.15	7.32 ± 0.04	8.72 ± 0.18
30	7.82 ± 0.04	7.74 ± 0.17	7.82 ± 0.04	7.59 ± 0.22	7.48 ± 0.09	7.88 ± 0.35
40	7.93 ± 0.02	6.12 ± 1.23	7.70 ± 0.13	7.50 ± 0.1	6.97 ± 0.86	7.66 ± 0.19

*Distilled deionized water was autoclaved at 121°C for 12 min prior to use.

Figure 3.1. Quantitation of biofilm density produced by *Salmonella* serovars on glass coverslips immersed in TSB using modified crystal violet staining and de-staining procedure.

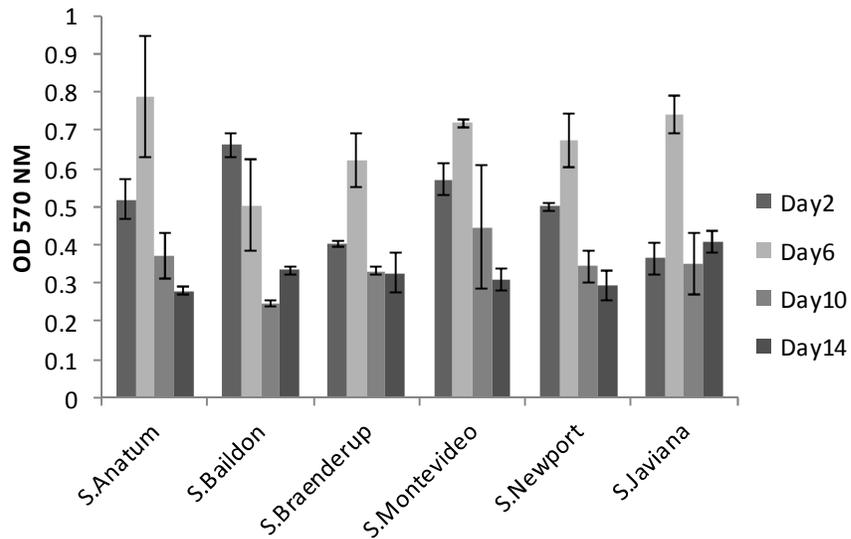


* Dye was assayed quantitatively for optical density at 570 nm after destining bound CV from coverslips using 33% acetic acid.

* Values account for inherent binding of CV by cover slip which resulted in a baseline mean residual OD of 0.09 at 570 nm.

* Error bars represent standard error of triplicates

Figure 3.2. Quantitation of biofilm density produced by *Salmonella* serovars on quartz particles immersed in TSB using modified crystal violet staining and de-staining procedure.



* Dye was assayed quantitatively for optical density at 570 nm after destaining bound CV from quartz particles using 33% acetic acid.

* Values do not account for inherent binding of CV by quartz resulting in a baseline mean residual OD of 0.46 at 570 nm.

* Error bars represent standard error of triplicates.

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CHAPTER 4. EFFECT OF HEAT SHOCK TO IMPROVE TRANSFORMATION IN TOMATO OUTBREAK ASSOCIATED *SALMONELLA* SEROTYPES AND COMPARISON OF BIOLUMINESCENCE EXPRESSION OF TRANSFORMANTS

Abstract

Bioluminescent *Salmonella* serotypes could serve as effective tools in real time imaging and produce safety research. *Salmonella* cells can be made bioluminescent by transforming them with *lux* operon containing plasmid. Outbreak or environmental isolates of *Salmonella* serotypes might often not be competent for transformation due to barriers produced by the restriction enzyme system of the recipient. We demonstrated that heat shock of cells after transformation was highly effective in overcoming barriers associated with xenogeneic DNA expression. We used heat shock to treat *Salmonella* cells transformed with plasmid containing *lux* operon. Heat shock treatment was highly effective in resulting in successful transformants ($P < 0.0001$). Absence of heat shock after transformation resulted in transformants that were not expressing bioluminescence. We also demonstrated that *Salmonella enterica* serotype Newport was most efficient in *lux* plasmid expression and that antibiotic selective pressure is essential for luminescence expression in *Salmonella* serotypes ($P < 0.0001$). The use of heat shock method to facilitate successful transformation in *Salmonella* cells is a novel approach.

Introduction

Light emission through bioluminescence is the result of a chemiluminescent reaction between a luciferin and luciferase. The *lux* A and B genes code for luciferases while the *lux* C, D and E genes code for proteins that generate its substrate (5, 11). The complete operon can be vectored into *Escherichia coli* or *Salmonella enterica* serotypes facilitating bioluminescence without substrate requirement hence making it an effective tool for *in vivo* studies (4).

Salmonella outbreaks have been associated with tomato consumption (7). While risk factors for produce contamination have been determined, routes through which contamination can occur require better understanding (1). Bioluminescent outbreak associated *Salmonella* serotypes could serve as a tool for the evaluation of plant-pathogen interaction. Bioluminescent imaging can be used to study and visualize bacterial pathogens in real time (5). Unlike fluorescence, the process is non-invasive and does not require excitation. Biophotonic imaging has been used to track pathogens in sprouts (15). Fluorescence imaging of produce might induce significant auto-fluorescence and reduce the sensitivity of the image modality. Using fluorescence to study internalization of *Salmonella enterica* in tomato tissues might require excising fruit for excitation of the fluorescent protein tagged pathogen (6). Cutting of fruit tissue could result in displacement of bacteria due to mechanical action of the cutting implement (10). The advantages of whole fruit imaging make bioluminescent *Salmonella* serotypes an important tool for produce safety research. Using bioluminescent outbreak associated *Salmonella* serotypes could create better paradigms for the study of plant-pathogen interaction testing.

Real-time pathogen monitoring and imaging requires understanding luminescence expression differences amongst serotypes. Determining plasmid stability with and without antibiotic selective pressure would also help in optimal experimental design. Electrotransformation is a commonly used technique to develop bioluminescent strains of bacteria for research. *E.coli* and *Salmonella* spp. are not naturally competent for transformation and *Salmonella* spp. have been known to yield lesser transformants per μg of DNA used. *Salmonella enterica* subsp. *enterica* serovar Typhimurium, has been reported to be untransformable by electroporation or transformable only at comparatively low levels (12). It was reported by Bautista et al. that only one of the seven *Salmonella* isolates from a poultry

facility were successfully transformed with the *lux* plasmid in their study to determine the long term effects of lactic acid rinse on pathogen viability and recovery . van der Rest et al. found that the efficiency of electrotransformation with xenogeneic DNA is severely limited by the restriction enzyme system of the recipient. They demonstrated that a heat shock treatment for 6 min at 46°C could improve electro-transformation efficiency during transfer of DNA between different bacterial species (14).

Our aim was to develop bioluminescent *Salmonella* serovars as a tool to study produce-pathogen interaction through real time imaging. We also wanted to determine the robustness and stability of bioluminescent strains for better experimental design. We tested the efficiency of heat shock to overcome the barriers of xenogeneic DNA transformation of *Salmonella* serovars associated with tomato outbreaks. Two different heat shock procedures were developed for *Salmonella* serotypes and compared. We also compared bioluminescence expression of the serotypes on quartz dust to determine highest suitability for *in vivo* and environmental studies. Plasmid stability in presence and absence of antibiotic over time was determined.

Materials and Methods

Bacterial strains

Stock cultures of *Salmonella enterica* subsp. *enterica* serovar Newport, Javiana, Montevideo, Anatum, Baildon and Braenderup were obtained from the culture collection at Virginia Polytechnic Institute and State University. Frozen stock cultures were thawed and a loopful from each stock culture was transferred to separate 25 ml of Brain Heart Infusion broth (BHIB; Becton, Dickinson and Co, Sparks, MD) and incubated for 24 hrs at 37°C. One loopful of culture was transferred thrice at intervals of 24 hrs into 25 ml of BHIB and incubated at 37°C for 24 hr in a shaking incubator to facilitate complete revival. A loopful of cells from the final

transfer into BHIB was streaked onto xylose lysine Tergitol 4 (XLT4); (Becton, Dickinson and Co, Sparks, MD) for presumptive determination of typical colonies and on Tryptic soy agar (Becton, Dickinson and Co, Sparks, MD) for growth on non-selective medium. The plates were incubated for 24 hrs at 37°C. Representative, cultures from the TSA plate was confirmed biochemically using 20E API strips (bioMerieux, Hazelwood, MO) and latex agglutination assay (Oxoid). Upon confirmation, colonies were streaked on TSA slants and incubated for 24 hrs at 37°C, following which the slants were stored in the refrigerator at 4°C for inoculum preparation.

Bioluminescent Salmonella strains

Competent cells were prepared by inoculating 45 ml of LB broth (Becton, Dickinson and Co, Sparks, MD) with 1 ml of an overnight culture of each *Salmonella* serotype. Once the optical density (OD) reached 0.8 at 600 nm, the cells were placed in ice for 15 min. The culture was centrifuged to pellet the cells and the supernatant was discarded. The pelleted cells were washed thrice with 15% ice cold glycerol to make them competent and stored in the deep freezer until use. Competent *Salmonella enterica* strains were transformed with broad host range plasmid pNSTr*lux* containing the *lux CDABE* operon (13). For electroporation, competent cells were placed on ice and then 40 µl of competent cells were transferred to a sterile eppendorf tube along with 1µl (50 ng) of plasmid DNA isolated from *E. coli* DH10B™ T1 using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The cells and plasmid DNA were mixed and spun down following which the mixture was transferred to an ice-chilled 2mm gap cuvette (Bio-Rad, Hercules, CA). Electrotransformation protocols were adapted from Howe et al.(8). For *Salmonella* Newport, *S. Baildon*, *S.Javiana* and *S. Anatum* following electroporation conditions used were, 2.5 kV, 25 µF and 400Ω using the Gene Pulser II system (Bio-Rad, Hercules, CA). For serotypes *S. Braenderup* and *S. Montevideo* electroporation conditions used were 1.8 kV, 25

μF and 600Ω . After electroporation, the cells were transferred into 450 μl SOC medium (2% Bacto Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl_2 , 10 mM MgSO_4 , 20 mM glucose) at 37°C in a shaking incubator at 100 rpm for 1 hour. For selection of transformants, 100 μl of the broth was spread plated on TSA plates containing chloramphenicol ($30\ \mu\text{g ml}^{-1}$) and observed for transformants after 24 hours of incubation.

Heat shock procedure

Two protocols of heat shock were tested on *Salmonella* serotypes- Newport, Javiana, Montevideo, Anatum, Baildon and Braenderup. After electrotransformation, cells were transferred into preheated SOC medium that had been incubated in a water bath at 46°C . In a scale-down heat shock procedure, post electrotransformation, cells were transformed into 450 μl of preheated SOC medium and incubated at 46°C for 1 min. In a scale-up heat shock procedure, post electroporation, cells were incubated in 4 ml of SOC medium for 6 min. Following heat shock procedure, 100 μl of the SOC broth was spread-plated on TSA plates containing chloramphenicol ($30\ \mu\text{g ml}^{-1}$) and incubated for 37°C for 24-48 hours.

Bioluminescence comparison

A colony of each bioluminescent *Salmonella enterica* serotype- Baildon, Braenderup, Newport and Javiana was individually inoculated into 45 ml of TSB containing chloramphenicol ($30\ \mu\text{g ml}^{-1}$) and incubated for 24 hours. The culture suspension was centrifuged at 1400 g for 6 minutes to pellet cells. The supernatant was discarded and pelleted cells were washed with sterile deionized distilled water containing chloramphenicol ($30\ \mu\text{g ml}^{-1}$) thrice. The cells were suspended in 45 ml of sterile distilled deionized water containing chloramphenicol ($30\ \mu\text{g ml}^{-1}$) and 100 μl of the water- *Salmonella* suspension was used to inoculate wells containing quartz

dust (-50-70 Mesh, Sigma Aldrich) of a 96 well plate (Corning 3790, Corning, NY) . This was repeated every day to image expression of bioluminescence over time. To test the stability of the plasmid without antibiotic pressure, another 96 plate well containing quartz particles was inoculated with *lux* containing transformants without the presence of antibiotic in inoculum. *S. Newport lux* was used as a positive control and untransformed *S. Newport* was used as a negative control as background studies had suggested that the transformed serovar had good luminescence expression, hence enabling an appreciable contrast for visual reference. *Lux* transformed *Salmonella enterica* serotypes, *S. Braenderup*, *S. Baildon*, *S. Javiana* and *S. Newport* harboring pNSTr*lux* were compared for bioluminescence production on inoculated quartz particles for a period from day 0 to day 7. Plates were incubated at 30°C. Plates containing bioluminescent *Salmonella* serotypes-quartz suspension were imaged using an EMCCD camera (Andor Technologies) after a 10 min incubation time in dark. The luminescence intensity per pixel from each well was scaled using ImageJ software.

Statistical analysis

The experiment for the studies was a randomized blocked design factorial treatment arrangement, repeated measures with sampling, blocked on replication. To determine the efficiency of 1 min duration and 6 min duration heat-shock treatments in producing bioluminescent transformations of *Salmonella* serotypes, Chi-square analysis, using the FREQ procedure of SAS 9.1 (SAS Inst., Inc., Cary, NC, USA) was used. Bioluminescence performance of *Salmonella* serotypes – *S. Baildon*, *S. Braenderup*, *S. Newport* and *S. Javiana* from a period of day 0 to day 7 was analyzed using PROC GLM. Significant differences among treatment groups were determined by a Tukey's honestly significant difference (HSD) test. Variables for

treatment, serotype, day and their interactions were included in the model. A probability value of less than 0.05 indicated statistical significance.

Results

Heat shock post transformation

Transformation of *Salmonella* serotypes with *lux* plasmid (pNSTrclux) isolated from *E. coli* resulted in the absence of transformed colonies or the presence of non-bioluminescent transformants exhibiting antibiotic resistance. A, 1 min and 6 min protocol of heat treatment of electrotransformed cells in SOC broth was tested to overcome the barriers associated with xenogeneic plasmid transformation. Both treatments resulted in presence of transformants expressing luminescence on TSA plates containing antibiotics for all serotypes except *S. Montevideo* (Table 4.1). Heat-shock treatments were highly significant ($P < 0.0001$) in producing successful bioluminescent transformants after the transformation process. Serotypes- Baildon, Braenderup, Newport, Javiana yielded bioluminescent transformants at dilution of 10^{-5} per μl (50 ng) of plasmid DNA. *S. Anatum* yielded bioluminescent transformants only at dilutions of 10^{-1} per μl (50 ng) of plasmid DNA. Accurate visual enumeration was hindered by presence of non-luminescent colonies near auto-luminescent colonies and differences in luminescence intensity emitted between colonies. Positive colonies were isolated from plates and confirmed for luminescence by streaking on antibiotic containing plate. Difference in luminescence expression between serotypes was observed. Hence we graded treatments for their ability to result in bioluminescent transformants. Heat shock for one minute resulted in 61.1% of the transformed serotypes to produce bioluminescence. While heat shock for 6 minutes resulted in 77.8% successful transformants of *Salmonella* serotypes. Heating for 1 min was not as significant ($P =$

0.08) as heating for 6 min ($P<0.01$) while no heating did not result in a significant increase in bioluminescent transformants ($P=0.47$).

Effect of selective pressure on bioluminescence expression of Salmonella serotypes

The mean population of *Salmonella* serotypes in quartz dust was 8.74 log CFU/g quartz particle on the day of inoculation. Bioluminescence expression performance between *Salmonella enterica* serotypes over a period from 7 days was tested in presence of antibiotic associated selective pressure. The microtiter well plates were imaged at the end of the 7 day period using an EMCCD camera (Figure 4.1). Analysis of flux revealed differences in bioluminescence patterns occurred between *Salmonella* serotypes. - Baildon, Braenderup, Newport and Javiana (Figure 4.1, Figure 4.2, Figure 4.3). The combined effect of antibiotic selective pressure with time was highly significant in affecting bioluminescence production by *Salmonella* serotypes tested. The effect of time on bioluminescence expression was highly significant ($P< 0.05$). No bioluminescence was expressed by any of the serotypes on days 6 and 7. On day 0, *S. Newport* and *Javiana* were significantly different in bioluminescence expression than *S. Baildon* and *S. Braenderup* ($P<0.05$). On day 1, *S. Newport* was significantly different than all the other serotypes in bioluminescence production on quartz particles ($P<0.05$) (Figure 4.1, Figure 4.3). This trend repeated till day 5 until no significant difference could be observed between all four *Salmonella* serotypes. Differences in plasmid stability were not observed between serotypes (Figure 4.1, Figure 4.3). No bioluminescence was observed on day 6 and day 7 for any of the serotypes. From Figure 4.3, it can be observed that *S. Newport* and *S. Javiana* were had higher expression of bioluminescence.

Effect of absence of selective pressure on bioluminescence expression of Salmonella serotypes

The effect of antibiotic was highly significant in affecting bioluminescence expression in *Salmonella* serotypes ($P < 0.0001$). No luminescence was observed on day 1 while luminescence was observed until day 5 with antibiotic selective pressure. Bioluminescence emission by *Salmonella* serotypes was observed after quartz inoculation only on day 0 (Figure 4.2). Similar patterns of bioluminescence expression were observed on day 0 in both experiments.

Discussion

Our aim was to develop bioluminescent *Salmonella enterica* serotypes for real time imaging of tomatoes. *Salmonella* serotypes can be transferred to tomato plant tissue (phyllosphere and rhizoplane) from contaminated soil (6). The pathogen can also survive in fallow soil for a period of six weeks while retaining their ability to attach and contaminate tomato plant tissue (1).

Bioluminescent tagging of bacterial pathogens can be done by the integration of the operon into the chromosome or by the use of plasmid vectors. Both these techniques have advantages. Integrating the *lux CDABE* operon into the chromosome reduces the amount of light produced and requires higher number of cells for detection. Transforming the plasmid through electro-transformation or conjugation could result in more light production due to the high copy number of the plasmid (3).

Initial attempts at electrotransformation of *lux* plasmid from *E.coli* to outbreak associated *Salmonella* serotypes did not result in bioluminescent transformants. All transformants obtained were expressing antibiotic resistance but not bioluminescence. It was reported by Bautista et al. that only one of the seven *Salmonella* isolates from a poultry facility were successfully

transformed with the *lux* plasmid in their study to determine the long term effects of lactic acid rinse on pathogen viability and recovery (2).

van der Rest et al. found that the efficiency of electrotransformation with xenogeneic DNA is severely limited by the restriction system of the recipient. They demonstrated that a heat shock treatment could improve electro-transformation efficiency during transfer of xenogeneic DNA (foreign DNA) into *Corynebacterium glutamicum* (14). To our knowledge, the effect of heat shock in overcoming barriers associated with xenogeneic DNA transformation has not been explored. We decided to test the efficiency of the heat shock treatment using two different volumes of SOC medium for 1 min and 6 min on *Salmonella* serotypes. Both treatments resulted in successful transformation of cells and presence of bioluminescent colonies. The 6 min heat shock treatment was more effective in aiding successful transformation ($P < 0.05$) (Table 4.1).

Bioluminescent colonies were present in close proximity to non-bioluminescent with differences in luminescence between colonies, making accurate enumeration challenging. Colonies that appeared to be bioluminescent were isolated and streaked on antibiotic agar before visual confirmation. Also plasmid isolated from the bioluminescent colony resulted in high frequency of bioluminescent transformants (unpublished data). This might have resulted from the plasmid being syngenic. Once we obtained bioluminescent serotypes of *Salmonella* we compared them for expression of bioluminescence and stability of plasmid over time (Figure 4.1, 4.2). Luminescence can be lost when antibiotic associated selective pressure is absent in the environment (4).

As much as a 10 fold difference in bioluminescence production between *Salmonella* serovars has been observed (9). Our studies revealed that *S. Newport* produced the highest luminescence followed by *S. Javiana*, *S. Baildon* and *S. Braenderup* (Figure 4.3). All serotypes

were significantly different in bioluminescence production over time ($P < 0.05$). *S. Newport* had better luminescence expression in comparison to 11 other serovars in a study by Karsi et al. (9).

Variation in *lux* plasmid stability has been observed between serovars (9). The combined effect of antibiotic selective pressure and time was highly significant in bioluminescence production by *Salmonella* serotypes tested ($P < 0.0001$). Selective pressure was essential for expression of luminescence for extended time period (Figure 4.2). No luminescence was detected from any of the serotypes at day 0 without antibiotic selective pressure (Figure 4.2). Variation in plasmid stability was not observed amongst the tested serotypes.

The use of heat shock to improve xenogeneic plasmid DNA transformation into *Salmonella* serotypes could help in more efficient transformation of xenogeneic DNA in outbreak associated Salmonellae or environmental isolates. Our results indicate that heat shock post electrotransformation could improve the efficiency of electrotransformation in *Salmonella* serotypes that are traditionally not competent. We also demonstrated that *S. Newport* transformed with *lux* plasmid produced the highest luminescence and that selective pressure through antibiotic is important for maintenance of luminescence over extended duration of time. These bioluminescent serotypes, associated with tomato related outbreaks can help shed light on survival strategies and adaptations in real time.

Table 4.1. Effect of 1 min and 6 min Heat Shock treatment at 46°C on transformation efficiency in *Salmonella* serovars at 46°C

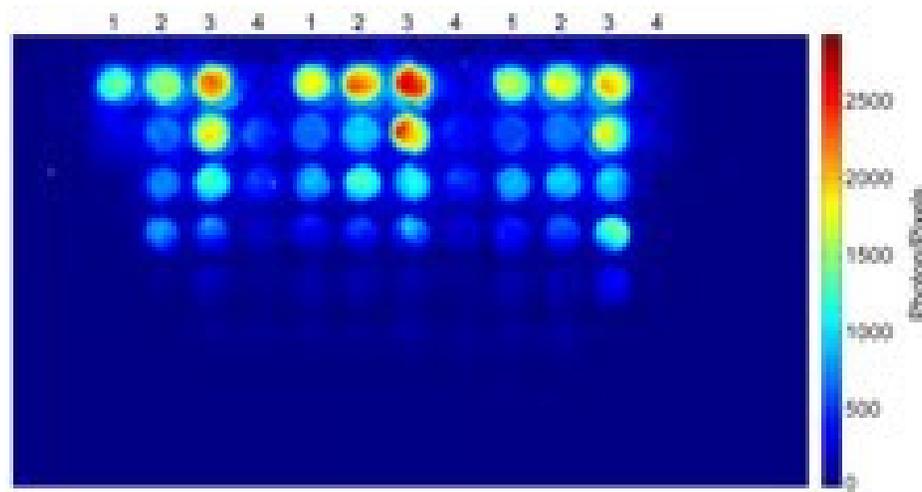
<i>Salmonella</i> serovar	Transformation	Transformation	Transformation
	efficiency (n=3)*	efficiency (n=3)	efficiency (n=3)
	1 Min*	6 Min*	No Heat
<i>S. Anatum</i>	2/3	3/3	1/3
<i>S. Baildon</i>	2/3	3/3	0/3
<i>S. Braenderup</i>	3/3	3/3	0/3
<i>S. Montevideo</i>	0/3	0/3	0/3
<i>S. Newport</i>	3/3	3/3	1/3
<i>S. Javiana</i>	1/3	2/3	0/3

*Transformation efficiency was determined by the number of transformation attempts (n=3) that yielded bioluminescent *Salmonella* serovars

* 1Min- Cells were heat shocked in 450 µl of preheated SOC medium and incubated at 46°C for 1 min.

* 6 Min- Cells were incubated in 4 ml of SOC medium for 6 min.

Figure 4.1. EMCCD camera image of bioluminescent *Salmonella* serovars with luminescence intensity scale (photon/pixel) over a period of 0-7 days on quartz particles (400 mg/well) under antibiotic selective pressure ($30 \mu\text{g ml}^{-1}$ of chloramphenicol).

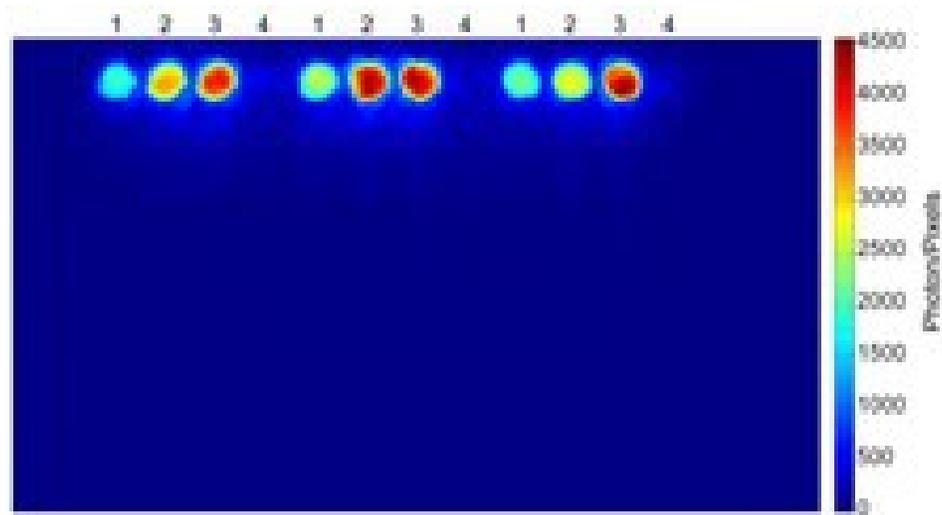


1-*S. Baildon*, 2- *S. Javiana*, 3- *S. Newport*, 4- *S. Braenderup*

* Rows on microtiter plate represent day 0 to day 8

* Columns on microtiter plate represent *Salmonella* serotypes Baildon (1), Javiana (2), Newport (3), Braenderup (4). Serotypes were imaged in triplicate.

Figure 4.2. EMCCD camera image of bioluminescent *Salmonella* serovars with luminescence intensity scale (photon/pixel) over a period of 0-7 days on quartz particles (400mg/well) without antibiotic selective pressure.

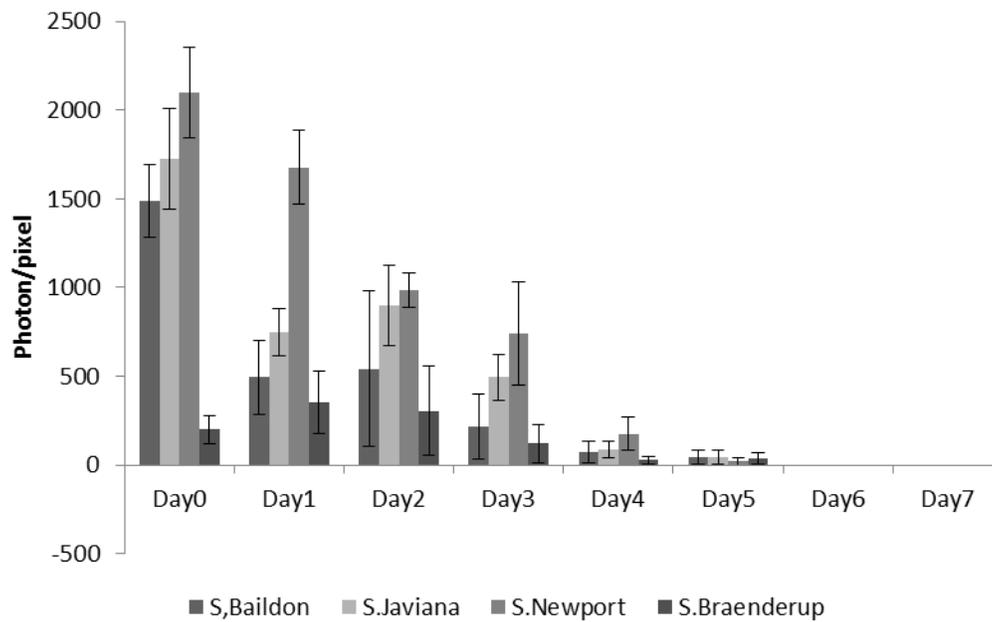


1-*S. Baildon*, 2- *S. Javiana*, 3- *S. Newport*, 4- *S. Braenderup*

* Rows on Microtiter plate represent day 0 to day 8

* Columns on Microtiter plate represent *Salmonella* serotypes *Baildon* (1), *Javiana* (2), *Newport* (3), *Braenderup* (4). Serotypes were imaged in triplicate.

Figure 4.3. Comparison of luminescence production (photon/pixel) by *Salmonella* serovars over a period of 0-7 days on quartz particles (400 mg/well) under antibiotic selective pressure (30 $\mu\text{g ml}^{-1}$ of chloramphenicol).



*Error bars represent standard deviation of luminescence readings performed in triplicate.

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CHAPTER 5. POTENTIAL OF AIRBORNE SOIL PARTICULATE TO SERVE AS VEHICLE FOR *SALMONELLA* IN TOMATO CONTAMINATION

Abstract

Salmonella can survive in soil and establish persistence in the farm environment. The potential of wind distributed soil particulate in contaminating tomato blossoms and consequentially the fruit was examined. Through studies using farm soil contaminated with *S. Newport*, it was demonstrated that soil particulate could serve as a vehicle of *Salmonella* resulting in the contamination of tomato blossoms ($P < 0.0001$). Soil particulate contaminated blossoms developed into fruit that were positive for the presence of *S. Newport*. Fruit and calyx tissue were positive for *S. Newport* even after surface sterilization suggesting presence in subsurface tissue. *S. Newport* cells in internal tomato tissue required enrichment for isolation. This study indicates that airborne soil particulate could serve as a vehicle for *Salmonella* if contaminated with the pathogen and that contact of contaminated dust with blossom could result in tomato fruit contamination. Natural Aeolian phenomenon in fields might contribute to transfer of pathogens and contamination of fruit indicating an omnipresent yet unexplored contamination route. Biophotonic imaging revealed that even momentary contact of *Salmonella* containing dust with blossoms could result in transfer of pathogen to blossom surface. Airborne soil particulate contact with blossoms resulted in fruit contamination suggesting that presence of *Salmonella* in agricultural soil could serve as a risk factor in tomato contamination since soil particles can potentially be dispersed due to air currents in the field environment.

Introduction

Tomato associated *Salmonella enterica* outbreaks have occurred more than a dozen times over the last two decades in the United States (5, 11). This has resulted in illness and financial

burden to the affected individuals and losses to tomato cultivators (11). *Salmonella enterica* is the leading cause of foodborne illness in the US and tomatoes are the most frequently associated produce item in *Salmonella* outbreaks (1).

Traceback investigations after outbreaks have indicated that contamination of tomatoes might occur on fields or in the packing house but the actual contamination route remains ambiguous (8). While sanitizing procedures might be effective in reducing surface load of pathogen, internalized *Salmonella* populations are not eliminated (18).

The internalization of *Salmonella enterica* inside tomato tissue has been achieved in laboratory conditions by brushing the blossom with inoculum and vacuum mediated internalization (9, 17). Internalization into subsurface tissue has also been demonstrated by mediating contact of fruit with contaminated soil (10). Temperature difference between fruit and dump tank water can result in contaminated water being internalized into fruit tissue through the stem scar (18).

Contamination routes in the field environment remain ambiguous and some of methods used in laboratory studies might not represent contamination routes of tomatoes grown on the field (3). Barak and Liang demonstrated that *Salmonella enterica* serotypes associated with tomato outbreaks could survive in soil for a period of six weeks and contaminate tomato plants (3). Traceback investigation of *Salmonella enterica* subsp. *enterica* serotype Newport associated tomato outbreaks revealed that the causative strain was isolated from irrigation pond water. Pond water did not come in direct contact with fruit and entered the soil bed (8) indicating possible presence in soil. Miles et al. investigated *S. Montevideo* internalization into fruit from contaminated irrigation water and seed stock. Fruits, roots, stem and leaves were sampled. None of the fruit sampled was positive for *S. Montevideo* after surface sterilization (14). *Salmonella*

enterica have been demonstrated to survive on plant tissue and fruit surface. Natural routes of pathogen entry into fruit and sources of fruit contamination need to be explored. Investigation of pathogen internalization into fruit tissue is necessary as 5 billion pounds of raw fruit is consumed every year in the United States.

The field environment is subject to Aeolian processes (wind based transport of sediments) and climatic changes. The potential of *Salmonella* contaminated soil or dust to serve as vehicles in transportation of pathogen from agricultural reservoirs to plant surface needs to be explored. A correlation between *Salmonella* presence and airborne particulates has been shown (13). *Salmonella* has also been isolated from air samples immediately post flushing indicating possible airborne transmission (4). Airborne soil particulates are omnipresent in the field environment (15). Soil particulate moved by wind can range up to about 1mm in diameter (19) indicating that the pathogen can attach to the surface of soil and the potential for air mediated dispersal exists.

The objectives of this study were to test the potential of soil particulate in delivering *S. Newport* to tomato blossoms. Both culture based techniques and real time imaging were used to gain a clearer understanding of pathogen transmission to tomato blossoms by soil particulate. The potential of soil particulate contaminated blossoms to develop into fruit containing *S. Newport* in internal tissue was also examined.

Materials and Methods

Bacterial strains

Stock cultures of *Salmonella enterica* subsp. *enterica* serovar Newport was obtained from the culture collection at Virginia Polytechnic Institute and State University. Frozen stock cultures were thawed and a loopful from each stock culture was transferred to separate 25 ml of

Brain Heart Infusion broth (BHIB; Becton, Dickinson and Co, Sparks, MD) and incubated for 24 hrs at 37°C. One loopful of culture was transferred thrice at intervals of 24 hrs into 25 ml of BHIB and incubated at 37°C for 24 hr in a shaking incubator to facilitate complete revival. A loopful of cells from the final transfer into BHIB was streaked onto a xylose lysine Tergitol 4 (XLT4); (Becton, Dickinson and Co, Sparks, MD) for presumptive determination of typical colonies and on Tryptic soy agar (Becton, Dickinson and Co, Sparks, MD) for growth on non-selective medium. The plates were incubated for 24 hrs at 37°C. Representative, cultures from the TSA plate was confirmed biochemically using 20E API strips (bioMerieux, Hazelwood, MO) and latex agglutination assay (Oxoid). Upon confirmation, colonies were streaked on TSA slants and incubated for 24 hrs at 37°C, following which the slants were stored in the refrigerator at 4°C for inoculum preparation.

Bioluminescent Salmonella Newport

Competent *S. Newport* cells were prepared by inoculating 45 ml of LB broth (Becton, Dickinson and Co, Sparks, MD) with 1 ml of an overnight culture of *S. Newport*. Once the optical density (OD) reached 0.8, the cells were placed in ice for 15 min. The culture was centrifuged to pellet the cells and the supernatant was discarded. The pelleted cells were washed thrice with 15% ice cold glycerol to make them competent and stored in the deep freezer until use. Competent *S. Newport* was transformed with broad host range plasmid pNSTrclux containing the *lux CDABE* operon. For electroporation, competent cells were placed in ice and then 40 µl of competent cells were transferred to a sterile eppendorf tube along with 1µl of plasmid DNA isolated from *E. coli* DH10B™ T1 using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The cells and plasmid DNA mixture were mixed and spun down following which the mixture was transferred to an ice-chilled 2mm gap cuvette (Bio-Rad, Hercules, CA).

Electrotransformation protocols were adapted from Howe et al. (12). For *S. Newport*, electroporation conditions applied were, 2.5 kV, 25 μ F and 400 Ω using the Gene Pulser II system (Bio-Rad, Hercules, CA). After electroporation, the cells were transferred into 450 ml SOC medium (2% Bacto Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) at 37°C in a shaking incubator at 100 rpm for 1 hour. For selection of transformants, 100 μ l of the broth was spread plated on TSA plates containing chloramphenicol (30 μ g ml⁻¹) and observed for transformants after 24 hours of incubation.

Plant growth conditions

Bonnie “Sweet 100” sweet cherry tomato plants were purchased from a local store during the summer of 2011. Plants had not reached flowering stage when procured and were healthy and disease free. The plants were transferred to alcohol sanitized pots containing Miracle-gro potting mix which was negative for presence of *Salmonella* on XLT4 agar. The plants were irrigated with tap water on a daily basis and Miracle-gro Liquid All-Purpose Plant Food (Scotts Miracle-Gro Products, Port Washington, N.Y.) was used weekly as per manufacturer’s instructions. Once the plants reached flowering stage (2 weeks), they were transferred to a Thermo Scientific, Precision, Plant-Growth Incubator. Plants were grown at 25°C with 12 hour light and dark intervals and 70% humidity. They were irrigated on a daily basis with tap water. A drip tray was placed at the bottom shelf of the incubator to collect water droplets after watering and plant debris. Once blossom formation occurred, each peduncle of tomato blossoms was tagged with a labeled tape at the base stem for identification. A plastic sampling cup with its base cut was also placed around the flowers to prevent dispersal of inoculum to other plant tissues.

Preparation of soil

Soil was obtained from a tomato farm in the eastern shore. The sandy soil was passed through a No. 20 sieve (U.S.A Standard Testing Sieve) to obtain a uniform grain size. The soil was autoclaved to eliminate populations of existing microorganisms in the soil. Autoclaved soil was tested for the presence of sterility by performing an aerobic plate count. The soil sample was also screened for the presence of *Salmonella* spp. by plating on XLT4 agar. Ten gram soil aliquots was stored in a sterile falcon tube and stored at 4°C until use.

Preparation of Inoculum

A loopful of *Salmonella enterica* serotype Newport was inoculated into 45 ml TSB (Tryptic soy broth) and incubated in a shaking incubator for 24 hours at 37°C. The broth was then centrifuged at 1400 g for 10 minutes to pellet the cells. The supernatant was discarded and the cells were washed with sterile deionized distilled water twice to rid them of any remaining media. The washed cells were centrifuged and the supernatant was discarded. Ten grams of sterile soil (aw 0.92) was added to the pellet and the mixture was vigorously vortexed to create equal distribution of *S. Newport* in soil. The container with its lid open was placed in a desiccator containing CaSO₄ (Drierite™ Xenia, OH) to facilitate the drying process for 48 hours.

Brush inoculation

A sterile, moistened paint brush was used to apply dust particle- *S. Newport* mixture to healthy tomato blossoms. Care was taken not to dislodge blossoms during dust application. Petals, stamens and pedicel was brushed with soil from a 100 mg aliquot of inoculated soil to ensure contact with the entire surface area of the blossom.

Air inoculation

Hundred milligrams of 48 hour dried soil- *S. Newport* mixture was placed on the edge of a sterile spatula. Internal air pressure was increased in a Nalgene aerosol spray bottle (#2430-0200, Fisher Scientific, Pittsburgh, PA, USA). The nozzle of the sprayer and the dust were placed near the flower and pressurized air was released to deliver the dust on to flower surface. Care was taken to prevent errant distribution of dust by using collection cup around blossom bunches. Inoculation of flower was performed in sterile cabinet. For controls, autoclaved dust was air inoculated into a blossom bunch from the same plant and the flowers were tested. Care was taken to choose a blossom bunch at the top of the plant to avoid accidental inoculation.

Microbiological testing of flower

Flowers were tested one week after inoculation for the presence of *Salmonella Newport*. The inoculated blossom bunches were excised and each individual blossom was placed in a sterile Whirl-Pak bag (Nasco, Fort Atkinson, WI), containing 10 ml of sterile peptone diluent (0.1% peptone). The bag was then massaged to facilitate particulate dislodging, following which the wash water was streaked on the XLT4 agar. Plates were incubated at 37°C for 48 hours.

Microbiological testing of fruit

Fruits that were produced three weeks after flower inoculation were tested. The fruits were excised and placed in labeled Whirl-Pak bags and massaged to dislodge surface attached cells. Hundred microliters of the wash water was plated on XLT4 and incubated at 37°C for 24 hours. Washed fruits were analyzed for internalized *S. Newport*. Fruits were surface sterilized by washing in 10% bleach and surface drying followed by a wash with 70% ethanol. The ethanol washed fruit was dried under UV radiation for 1 hour. The surface sterilized fruits were then

placed in a whirlpak bag and macerated with a mortar and pestle to ensure exposure of the internal tissues to the enrichment medium. The macerated fruit was incubated overnight in 10 ml of buffered peptone water for 24 hours at 37°C. After incubation of the fruit in buffered peptone water 100 µl of the buffered peptone was plated on XLT4 and incubated at 37°C for 24 hours. Following this, 1 ml of the buffered peptone water in which the fruit had been incubated was then transferred to 10 ml of Tetrathionate broth. After 24 hours incubation at 37°C, 100µl of the tetrathionate broth was spread plated on XLT4. The XLT4 plates were incubated at 37°C for 24 hours. Black colonies indicative of *Salmonella* on XLT4 plates were considered positive. These were confirmed as *Salmonella* through latex agglutination test.

Preparation of inoculum for bioluminescent imaging

Dispersal of *Salmonella* cells on flower was also studied using bioluminescent *S. Newport* transformed the broad range pNSTrc-lux plasmid containing the *lux CDABE* operon. Inoculum was prepared by the same procedure described previously for blossom inoculation except that the media used to grow the *S. Newport* cells and the distilled deionized water used for washing the pelleted cells contained chloramphenicol (30 µg mL⁻¹) to aid in plasmid retention by *S. Newport* cells.

Inoculation and imaging

Inoculation was performed using pressurized air from a Nalgene aerosol spray bottle as described earlier. Once the blossoms were inoculated, they were excised and placed in a sterile petri dish for imaging. Imaging was performed using an electron-multiplying charge-coupled device camera (Andor, Ixon) 4 hours after inoculation.

Statistical analysis

The experiment of flower inoculation studies was a randomized complete blocked design. Only data from experiments conducted in triplicates was statistically analyzed. The effects of brushing and air delivery of inoculated dust on tomato blossoms were analyzed by Chi-square analysis and Fisher's exact test, using the FREQ procedure of SAS 9.1 (SAS Inst., Inc., Cary, NC, USA). Air inoculation of blossoms and resulting contamination of fruit was also analyzed by Chi-square analysis and Fisher's exact test, using the FREQ procedure. A probability value of less than 0.05 indicated a statistically significant.

Results

Brush Inoculation

Inoculation of *Salmonella* contaminated dust on tomato blossoms resulted in *S. Newport* presence on blossom tissue indicating that brushing of dust on blossom could result significant contamination (P=0.002). The population of *S. Newport* in dust for blossom inoculation was approximately 9.39 log CFU/g of dust. Blossoms were dust inoculated with a moistened brush. The petals, stamens and pedicel of the blossoms were all brushed to ensure an even distribution. Few flowers were dislodged during the process and these were discarded. Controls were brushed with dust that had been autoclaved. All flowers were tested after a week from inoculation. Eleven blossoms had dropped during the week after inoculation and these were discarded. Only blossoms that were healthy and attached to the peduncle were selected. Six blossoms were tested for the presence of *S. Newport* by spread plating agitated suspensions on XLT4 agar and looking for typical colonies. All 6 of the flowers tested positive for the presence of *S. Newport* and none of the controls were positive (Table 5.1).

Air Inoculation

Compressed air was used to mimic wind current to deliver dust on to blossom surface. Most of the dust particles had only momentary contact with the blossom. Some of the dust was trapped inside the blossom and could be seen adhered on the stamens and petals. After inoculation 7 blossoms were dislodged from the pedicel. These were discarded. The blossoms were tested using the same protocol as brush inoculated blossoms. Four out of the 14 blossoms tested (28.6%) were positive for presence of *S. Newport* (Table 5.1) 1 week after inoculation. None of the controls that had been air inoculated with autoclaved dust were positive for the presence of *S. Newport*.

Fruit internalization

Tomatoes at the immature green stage were tested for the presence of *S. Newport* to determine flower to fruit transmission. Fruits were tested by enrichment in buffered peptone water and tetrathionate broth. Fruits were formed within 2-3 weeks of air inoculation of blossoms and were tested. Fruit with intact calyx (IC), fruit without calyx (WC) and calyxes that had been excised from fruit were screened for the presence of pathogen both on the surface and after surface sterilization. A total of 25 fruits with intact calyx, 18 fruits without calyx (Table 5.2) and 9 calyxes were tested for the presence of *S. Newport*. Wash water was positive for *S. Newport* on one fruit with intact calyx and one excised calyx. Incubation of surface sterilized macerated fruit tissue in buffered peptone water for 24 hours resulted in 1 fruit (WF) and 1 calyx being positive for the presence of *S. Newport*. Enrichment in tetrathionate broth of surface sterilized fruit tissue resulted in 12/18 fruits (WF) and 7/11 calyx being positive for the presence of *S. Newport*. Fruits attached to calyx tissue (IC) were tested from one plant and 10 of these (n=25) surface sterilized fruits were positive for *S. Newport* after tetrathionate broth enrichment.

Samples from three separate plants were used to test whole fruit (WF) and calyxes separately for presence of *S. Newport* after surface sterilization (Table 5.2). Fruit and calyx formed from dust inoculated blossoms were positive for presence of *S. Newport*. None of the controls were positive for *S. Newport*.

Bioluminescent imaging

Three separate peduncles with open flowers were inoculated with dry dust- *S. Newport* inoculum through compressed air. Three blossoms that received the inoculation of bioluminescent *S. Newport*–dust mixture were selected. They were excised using a sterile scalpel and placed in a petri dish. Flowers were imaged using an EMCCD camera 4 hours after dust inoculation to study dust distribution pattern on blossom tissue. Bioluminescent *S. Newport* from dust was distributed in internal blossom tissue such as petals and the stamen (Figure 5.1). Contact of dust also resulted in distribution and attachment of *S. Newport* to the pedicel. Duration of contact with dust particles was approximately 2 seconds. No dust adhesion was observed during visual examination of flower tissue. All three blossoms from three different pedicles were positive for bioluminescent *S. Newport* after imaging.

Discussion

Salmonella internalization into tomato fruit has been achieved through techniques such as brushing of blossoms with inoculum, vacuum cycles and stem injection (16). While these methods have resulted in presence of pathogen on fruit surface and in tissue, sources of the pathogen or events resulting in fruit contamination on the field need to be determined. Understanding natural contamination routes could help reduce on-field contamination of fruit.

Salmonella serotypes can survive and replicate not only on the phyllosphere, rhizosphere and surface of fruit but also in internal tomato tissues (9, 16). The acidic environment of the fruit pulp does not provide an effective barrier to the pathogen (6). Surface disinfection of the fruit does not ensure a safe product for the consumer as internalization of the pathogen has been demonstrated. Various studies have demonstrated pathogen entry into internal fruit tissue (9, 10, 18) through blossoms though natural contamination routes of blossoms remain to be understood.

The role of *Salmonella* contaminated soil and water in transmission of pathogen has been examined but the role of airborne soil particulate in tomato contamination has not been explored even though soil particulate dispersal in fields is a common phenomenon (19). Chinivasagam et al. assessed the levels of *Salmonella* and *Campylobacter* in aerosols present within and outside poultry sheds. It was found that the levels of bacteria in the air are related to the level of the same bacteria in the litter. The *Salmonella* serotypes isolated in litter during the study were generally also isolated from aerosols and dust (7). Lues et al. studied the microbial composition of a high-throughput chicken slaughtering facility over a four month period. *Salmonella* serotypes were detected in the facilities. Highest microbial counts were found in the receiving killing and defeathering areas. A strong correlation was found between *Salmonella* spp. presence and airborne particulates. *Salmonella* spp. was rarely isolated from other processing areas indicating that the organism might be introduced into the facility by dust spread by the birds. Dust was the only environmental factor to have an influence of significance on the dispersal of *Salmonella* spp (13).

The airborne transmission of contaminated soil particulate and its potential in *Salmonella* transmission to blossoms was studied using culture based methodology and bioluminescent imaging. Dust is defined as fine particulate matter removed from land surface due to winds (15),

hence compressed air was used to mimic natural wind currents. The results show that *S. Newport*. can be transferred from contaminated dust to blossom and ultimately result in contamination of fruit surface and internal tissue. Imaging studies revealed that *S. Newport* was transferred both inside blossom and on the pedicel of the blossom. *S. Newport* was observed on the petals and on the stamen of the flower 4 hours after contact with dust. This might indicate certain tomato tissues might retain *Salmonella* spp. after contact. Retention of *Salmonella enterica* on surface tissue was observed even though the dust was dry and contact was momentary. Barak et al. reported that after dip inoculation, type 1 trichomes, fruit, calyx and pedicels of tomato plants could harbor *Salmonella* (2). Petals, sepals, pedicels, fruit surface, sruti tissue and calyxes were contaminated during blossom and consequential fruit contamination by soil particulate.

Internalization was defined as the presence of *S. Newport* on surface sterilized fruit tissue. *Salmonella* was isolated from fruit and calyx surfaces. The pathogen was also detected in surface sterilized calyx and fruit. The presence of pathogen after surface sterilization of fruit and calyx could indicate presence of pathogen in internal tissue. Most of the fruits tested positive for the presence of *S. Newport* after enrichment in tetrathionate broth suggesting cells might have endured stress during the internalization process and survival in immature fruit.

During our study, we noticed that brushing flowers with *Salmonella*-dust mixture also led to wilting and dropping of blossoms. This could have resulted from the mechanical stress of brush contact. Higher rates of humidity might have led to blossom drop. When a more natural path of contamination, such as air current was used to inoculate the flower, the levels of blossoms dropping reduced.

Biophotonic imaging of bioluminescent *S. Newport* contaminated blossoms indicated pathogen dispersal on petal, sepal, stamen and pedicel. Background studies indicated that plasmid stability was short lived (1 day) without antibiotic selective pressure in *S. Newport*. The development of a more stable bioluminescent serotype could be used in long term tracking of the pathogen in internal fruit tissue further elucidating pathogen-plant interactions. *Salmonella enterica* can survive in the soil environment. This study indicates that soil can also serve as a vehicle of *Salmonella* dispersal and that the pathogen can be transferred to blossom surfaces upon contact.

Blossom inoculation with *S. Newport* can result in its presence in internal fruit tissue. Methods to monitor and prevent accidental or unintentional inoculation of agricultural soil during flowering stage of tomatoes should be developed. *Salmonella* contamination of agricultural soil might be a risk factor especially during the blossoming stage of tomatoes.

Table 5.1. Effect of soil particulate inoculation on tomato blossoms with *S. Newport*/soil particulate mixture.

Method of Inoculation	Blossoms from Plant 1	Blossom from Plant2	Blossom from Plant 3	Total positive _c
Brush (n=6)	2/2	2/2	2/2	6/6
Air (n=14)	1/4	1/6	2/4	4/14
Control*-Brush (n=9)	0	0	0	0/9
Control*- Air (n=9)	0	0	0	0/9

* Blossoms were tested for *S. Newport*, one week after inoculation by spread plating wash water on XLT4 and confirming typical colonies thorough latex agglutination test

* Control blossoms from three plants were inoculated with sterile uninoculated soil using brushing.

* Control blossoms from three plants were inoculated with sterile uninoculated soil using compressed air delivery

Table 5.2. Microbiological analysis of fruit tissue, post tomato blossom inoculation with *S.* Newport/ soil particulate mixture..

Plant	Surface presence of <i>S.</i> Newport in tissue	Presence of <i>S.</i> Newport after 24 hour incubation in buffered peptone water	Presence of <i>S.</i> Newport after 24 hour enrichment in TT broth	Total number of positive samples <i>S.</i> Newport ^a
Calyx and fruit(n=25) _a	2/25	3/25	10/25	10/25
Fruit1 _b (n=6)	0/6	0/6	3/6	3/6
Fruit2 _b (n=6)	0/6	0/6	5/6	5/6
Fruit3 _b (n=6)	0/6	1/6	4/6	4/6
Total (n=43)				22/43
Calyx (n=11)	1/11	1/11	7/11	7/11
Control (n=9)	0/9	0/9	0/9	0/9

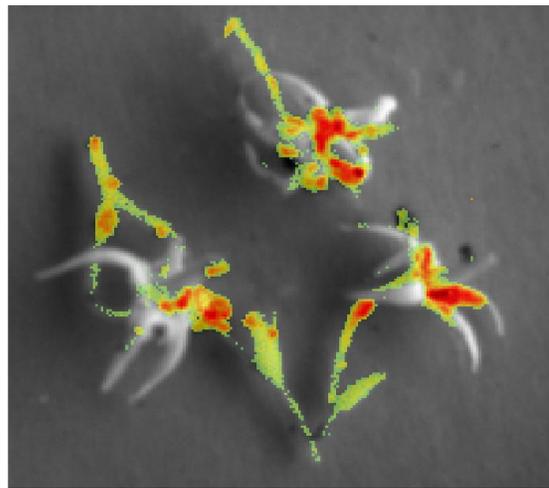
* Fruits were tested for *S.* Newport, one week after inoculation by spread plating wash water on XLT4 and confirming typical colonies thorough latex agglutination test

_a Fruit from plant was tested with the calyx intact.

_b Fruit from three separate plant were tested without the presence of calyx

_c Tetrathionate broth

Figure 5.1. EMCCD image of blossoms, air-inoculated with bioluminescent *S. Newport*/ soil particulate mixture.



*Location of dispersed *S. Newport* can be elucidated from colored portion of blossoms

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CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

Aeolian processes such as the wind mediated transfer of soil particles is commonplace in the farm environment. We determined the role airborne soil particulate in transfer of *Salmonella* spp. to tomato blossoms and its consequences. Survival and biofilm production studies of outbreak associated *Salmonella* serotypes revealed that dust mediated transfer of pathogen to blossom could be possible.

Our results indicate that *Salmonella* serotypes can survive in soil and water for a period of 40 days. We also noticed that there was no significant difference between survival patterns between serotypes. XLT4 agar was less sensitive in demonstrating variations in population over time than TSAYE. Hence using dual plating is an effective way to account for cell damage and survival patterns in soil.

All *Salmonella* serotypes tested were capable of rdar morphotype formation, necessary for abiotic surface attachment. The outbreak associated *Salmonellae* were capable of biofilm formation on both cover slips and quartz particles. The patterns of biofilm formed varied with material. Different trends in attachment were observed between serotypes.

During development of bioluminescent *Salmonella* serotypes for blossom inoculation experiments, we observed that outbreak associated strains might not be competent for electrotransformation. Restriction system associated barrier in *Salmonella* spp. could hinder acceptance of xenogeneic DNA in transformants. We demonstrated that heat shock was effective in overcoming the hurdle of xenogeneic DNA transfer in *Salmonella* serotypes.

Using bioluminescent serotypes of *Salmonella* and biophotonic imaging, we were able to demonstrate airborne dust and soil particulate associated transfer of *S. Newport* to blossom surface. We also demonstrated that when the contaminated blossoms bore fruit, the fruit was also

positive for the presence of *S. Newport*. This indicated that blossom and consequentially fruit contamination, could occur from contact with *S. Newport* containing soil particulate.

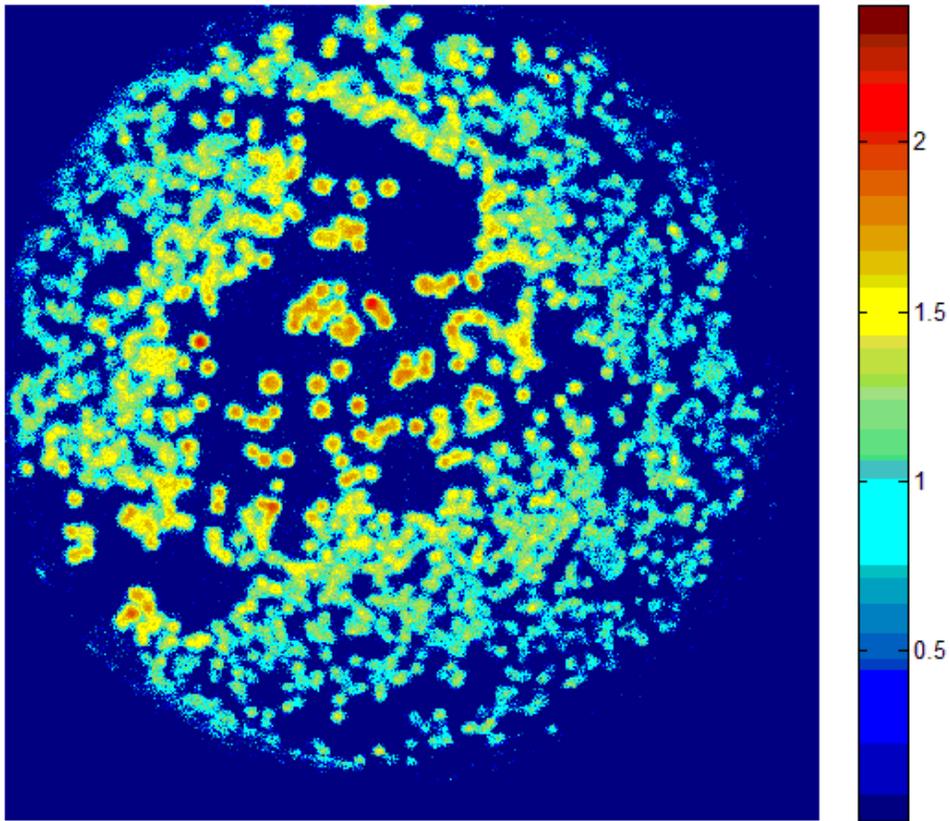
The study, while demonstrating a possibly common but unexplored, route of produce contamination also presents opportunities for more research.

Understanding survival of *Salmonellae* during composting and manure applications could help us determine routes and sources of contamination. It is also important to develop sensitive methods of enumeration since differences can occur between microbiological media. During our imaging studies, we determined that bioluminescence without antibiotic selective pressure in *Salmonellae* is short lived. Hence developing techniques to improve plasmid stability or chromosome integration is important. Contamination strategy of different *Salmonella* serotypes in tomato plants requires more understanding. Effective strategies for pathogen control in blossoms need to be explored. Bacteriophages, nematodes, predatory bacteria, antimicrobial sprays and sanitizing techniques should be tested to determine ability to prevent contamination of fruit while retaining desirable organoleptic qualities of fruit.

In conclusion, this study sheds light on a new possible route of contamination of tomato blossoms and fruit.

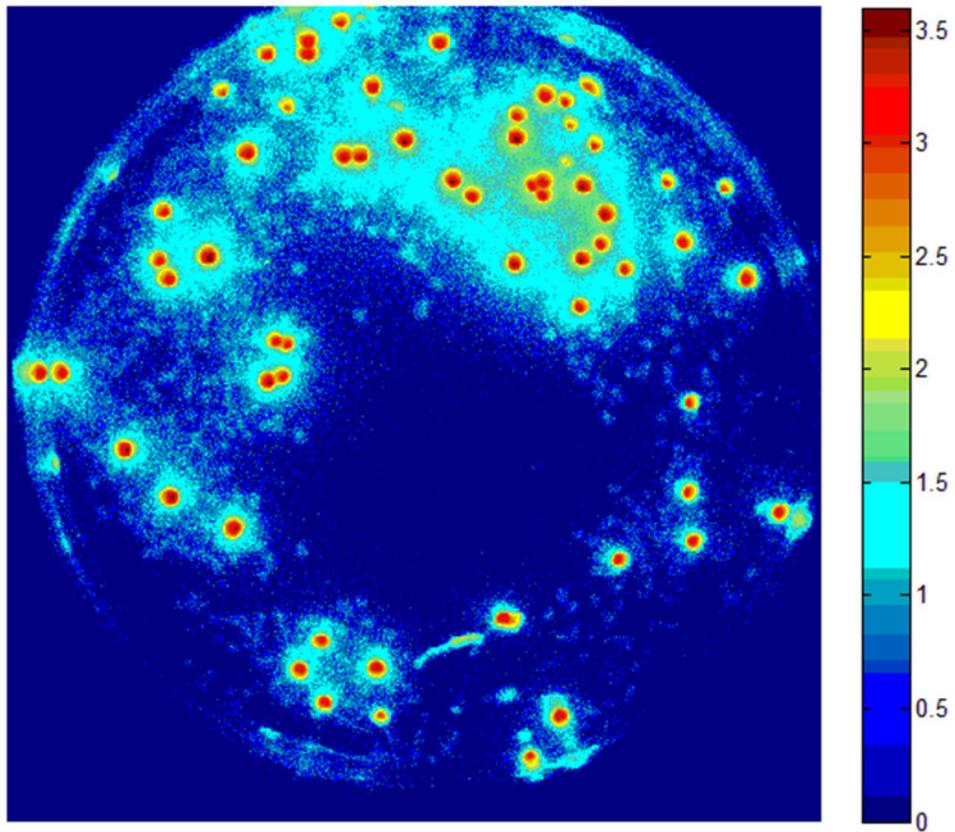
**APPENDIX A. COMPARISON OF BIOLUMINESCENCE EXPRESSION BETWEEN *S.*
ANATUM AND *S.* NEWPORT**

Figure A.1: EMCCD camera image of pNSTr*lux* transformed *S.* Anatum



Scale is in Photon/Pixel

Figure A.2: EMCCD camera image of pNSTr*lux* transformed *S. Newport*



Scale is in Photon/Pixel