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Migration, PCR Detection and Molecular Typing of *Salmonella* during Preharvest Production and Postharvest Storage of Produce Using Tomato as A Model
(Under the Direction of Dr. Larry R. Beuchat)

Salmonellae have been increasingly identified as a cause of outbreaks of infections associated with raw produce. However, lack of information on sources of contamination before and after harvest, location of cells on or in raw fruits and vegetables, and sensitive detection techniques make it difficult to develop efficient intervention steps.

A PCR assay derived from *hilA*, a positive regulator of *Salmonella* invasive genes, was developed for detecting the pathogen on tomatoes. The assay was tested on eighty-three *Salmonella* and twenty-two non-*Salmonella* strains, and validated for detecting *Salmonella* Montevideo in and on inoculated tomatoes; 10^2 and 10^1 CFU/g, respectively, were detected, using a 6-h enrichment at 37°C. Results indicate that the *hilA*-based PCR assay is sensitive and specific.

To investigate the establishment of *Salmonella* in tomato plants through different routes before harvest, a five-serotype mixture of *Salmonella* was used to inoculate plants, either by injecting stems or brushing flowers. Microbiological analysis of ripe fruits showed that 43 and 40% of stems inoculated before and after fruits set, respectively, were positive for *Salmonella*; 25% of fruits produced from inoculated flowers were positive.

Uptake of *Salmonella* by roots of tomato seedlings grown hydroponically was also studied using *Salmonella* tagged with green fluorescent protein. The pathogen was detected in cotyledons, stems, and leaves within 9 days of inoculation of nutrient solution in which cut and intact roots were submerged.

Salmonella survived for at least 45 days in inoculated moist soil. The population of *Salmonella* on tomatoes in contact with soil increased by 2.5 $\log_{10}$ CFU/tomato during storage at 20°C for 4 days, and remained constant for an additional 10 days. The pathogen decreased by ca. 4 $\log_{10}$ CFU/tomato during storage for 14 days at 70% relative

humidity. Imaging of cut tomatoes revealed that more cells were in the stem scar and subsurface areas of tomatoes as the time of storage in contact with inoculated soil increased. PCR fingerprinting showed that among five serotypes tested, *S.* Montevideo was the most persistent serotype detected on tomatoes.

This investigation demonstrates that tomato roots, stems, and flowers are possible sites at which *Salmonella* may establish and remain viable during plant growth and fruit development.

INDEX WORDS: *Salmonella*, Tomato, PCR, Migration, Survival

MIGRATION, PCR DETECTION AND MOLECULAR TYPING OF *SALMONELLA* DURING PREHARVEST PRODUCTION, POSTHARVEST STORAGE OF PRODUCE USING TOMATO AS A MODEL

by

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Fresh produce as a vehicle of human pathogens

In recent years, outbreaks of infections associated with raw and minimally processed fruits and vegetables have occurred with increased frequency (De Roever, 1999). The number of reported outbreaks of infections associated with fruits and vegetables in the U.S. more than doubled from 1973 to 1987 (4.3 per year) to 1988 to 1991 (9.75 per year) (Tauxe, 1997).

Raw and minimally processed fruits and vegetables are an essential part of people's diet around the world (Beuchat, 1998). In the past two decades, the consumption of fresh fruits and vegetables in the United States has increased, and the geographic sources and distribution of fresh produce have expanded greatly due to advances in agronomic processing, preservation, packaging, shipping, and marketing technologies on a global scale. However, some of these same technologies may also have brought an increased risk of human illness associated with a wide range of pathogens (Beuchat, 1997; Beuchat, 1998; Tauxe, 1997).

Among the factors possibly involved in changing the epidemiology of produce-associated disease, are changes in the food industry itself. Larger and more centralized production units and longer food chains could permit growth and distribution of pathogens to susceptible populations. The increased use of manure rather than chemical fertilizers may play a role, as improperly treated manure could introduce foodborne pathogens such as *Salmonella* and *E. coli* O157:H7. An additional factor is the growth in global trade, which makes food from around the world available to the average customer, bringing an end to seasonality in the food supply, and exposing consumers to exotic microflora. The trend toward more meals being eaten outside the home and the growing popularity of salad bars increases the risk of retail food handling error with fresh produce.

An increasing proportion of the population is elderly, immunocompromised, or suffers from chronic diseases (Hedberg, 1994; Tauxe, 1997). This population is at highest risk for severe outcomes from foodborne diseases.

Salmonellosis associated with fresh produce

Salmonella is one of the most prevalent foodborne pathogens in the U.S. It is estimated to cause approximately 1.5 million cases of infection, with 15,000 hospitalizations and 500 deaths annually (Mead, 1999).

Salmonella is facultatively anaerobic gram-negative rod belonging to the family Enterobacteriaceae. It includes over 2,300 serovars recognized by the Kauffmann-White scheme (D'Aoust, 1997). Human *Salmonella* infections range from mild, self-limiting inflammation of the intestinal mucosa to typhoid fever, a life-threatening systemic infection. Most infected people develop diarrhea, fever, and abdominal cramps 12-72 h after infection. The illness usually lasts 4 to 7 days, and most persons recover without treatment. However, in some people, diarrhea may be so severe that they need to be hospitalized. In these patients, the *Salmonella* infection may spread from the intestines to the blood stream, and then to other body sites, such as joints, irritation of eyes, and painful urination, which called Reiter's syndrome. Illness can last for months or years, and can lead to chronic arthritis that is difficult to treat. Newborns, infants, elderly, and those with impaired immune systems are more susceptible to *Salmonella* infections than healthy adults (D'Aoust, 1989).

In 1999, 10,697 laboratory-confirmed cases of foodborne disease were documented through national surveillance system (CDC, 2000). There were 4533 cases of foodborne salmonellosis; among the 4095 *Salmonella* isolates serotyped, 982 (24%) were serotype Typhimurium, 403(10%) were serotype Enteritidis (SE), 362 (9%) were serotype Newport, 284 (7%) were serotype Heidelberg, and 231(6%) were serotype Muenchen; 405 (10%) of *Salmonella* isolates were untyped. For all reporting sites in 1999, incidence per 100,000 population was highest for salmonellosis (17.7) (CDC, 2000). Historically, salmonellosis has most often been associated with consumption of contaminated food of

animal origin, such as poultry, eggs, meat, and dairy products. Changes in agronomic practices and dietary habits, and increased importation of fresh produce are thought to contribute to increased numbers of outbreaks associated with fruits and vegetables in recent years (Altekruse, 1997). Outbreaks of salmonellosis have been linked to tomatoes (CDC, 1993; CDC, 1999b; Wood, 1991), seed sprouts (Mahon, 1997; O'Mahony, 1990; Van Beneden, 1996), watermelons (Blostein, 1991; CDC, 1979; Gayler, 1955), cantaloupes (CDC, 1991; Ries, 1990), and unpasteurized apple cider (CDC, 1975) and orange juice (CDC, 1999a; Cook, 1996).

In 1990, two multistate outbreaks of salmonellosis associated with fruits and vegetables occurred: *S.* Chester associated with cantaloupes infected at least 245 persons in 30 states (Ries, 1990), and *S.* Javiana gastroenteritis associated with tomatoes affected 174 persons in four states (Wood, 1991). During June and July 1991, more than 400 laboratory-confirmed infections caused by *Salmonella* Poona associated with cantaloupe occurred in 23 states in the U.S. and Canada (CDC, 1991). In 1999, several large salmonellosis outbreaks were traced to produce or produce products as vehicles, including unpasteurized orange juice (*S.* Muenchen), mangos (*S.* Newport), and raw sprouts (*S.* Mbandaka) (CDC 1999). Consumption of fresh tomatoes was epidemiologically linked to 176 cases of *Salmonella* Javiana infection in Illinois, Michigan, Minnesota, and Wisconsin in 1990 (Wood, 1991). In 1993, tomatoes were identified as the vehicle for a multistate outbreak of *Salmonella* Montevideo infection (CDC, 1993). More recently, *Salmonella* Baildon was implicated in an outbreak with diced tomatoes in geographically separate areas of the U.S. (CDC, 1999b).

Zhuang et al. (1995) described conditions influencing survival and growth of *Salmonella* Montevideo on the surface of intact tomatoes. Rapid growth occurred in chopped ripe tomatoes (pH 4.1±0.1) at ambient temperature. *Salmonella* Enteritidis, *S.* Infantis, and *S.* Typhimurium were reported to grow in fresh cut tomatoes (pH 3.99 - 4.37) at 22 and 30^{o}C (Asplund, 1991). Wei et al. (1995) reported that *S.* Montevideo is able to multiply on wounded and cut tomatoes. The acidic pH (4.2 - 4.39 for ripe

tomatoes and 4.33 - 4.52 for green tomatoes) did not completely inhibit growth. Weissinger et al. (2000) reported that *S.* Baildon can grow in diced tomatoes (pH 4.40 ± 0.01); 0.79 $\log_{10}$ CFU/g increased to 5.32 and 7.00 $\log_{10}$ CFU/g within 24 h at 21 and 30^{o}C, respectively. However, after treatment with 200 µg/ml chlorine, diced tomatoes initially containing 0.60 - 0.86 $\log_{10}$ CFU of *S.* Baildon per g still harbored the pathogen (Weissinger, 2000). Chlorinated water is more effective in removing or inactivating S. Montevideo on tomato skin than in internal core tissue (Wei, 1995).

As an indicator of enteric bacteria, *Salmonella* has been isolated from horticultural crops or from wash waters (Wells, 1997). In Italy, Ercolani (1976) found *Salmonella* in wash water from 68% of the lettuce and 72% of the fennel samples tested. In the U.S., Rude et al. (1984) cultured *Salmonella* from 4 of 50 vegetables sampled. In Spain, Garcia-Villanova-Ruiz et al. (1987) cultured *Salmonella* in 7.5% of market vegetables samples, and incidence on various vegetable commodities has been reported in England (O'Mahony, 1990), Egypt (Saddik, 1985), Iraq (Al-Hidaw1,1979), and Italy (Fantasia, 1994). The U.S. Food and Drug Administration (FDA) examined imported cantaloupes and watermelons in 1990 and 1991 and isolated many serotypes of *Salmonella* from approximately 1% of the rinds. In contact with the ground, melons may be contaminated on their surface with dirt, chemicals, animal excreta, or bacteria, including *Salmonella* (CDC, 1991).

Wells et al. (1999) collected samples of fresh fruits and vegetables in the marketplace. They found that *Salmonella* contamination was present in at least 18-20% of soft rotted samples and in 9 to 10% of sound samples. Populations of bacteria were also affected by the presence of soft rot bacteria. However, produce infected with fungal rots or mechanically damaged had little or no greater than average risk of *Salmonella* contamination.

While it can be assumed that *Salmonella* and other fecal coliforms that may be present in commercially handled fresh produce are at base levels, any factors that favor multiplication of bacteria before consumption could result in a public health problem.

Routes of contamination of raw produce with pathogens

In the farm-to-table production, processing, and distribution chain, there are various possible points of contamination of fruits and vegetables with disease-causing microorganisms. These include irrigation water, runoff water from adjacent farms, manure, wash water, handling by workers, and contact with contaminated surfaces, animal manure used in previous growing seasons, or droppings from rodents or ruminants (Beuchat, 1997; Tauxe, 1997). It is essential that interventions be developed to prevent or minimize contamination of raw produce and to kill or remove pathogens prior to consumption. To date, however, none of the chemical or physical treatments currently authorized by regulatory agencies for use to disinfect raw produce can be relied on to eliminate all types of pathogens from the surface or internal tissues (Beuchat, 1998). One of the keys to enable the selection of appropriate intervention steps to reduce populations of pathogenic microorganisms on fruits and vegetable is to identify sources of contamination, and to characterize the ecology of pathogens as affected by agronomic and processing practices (Beuchat, 1998; Brackett, 1999; Buchanan, 1999).

One important source of fecal contamination appears to be related to the quality of water used for irrigation. It has long been known that the use of irrigation water with high number of enteric bacteria, viruses, protozoa, or helmiths results in increased frequency of pathogens on harvested produce (Norman, 1953; Dunlop and Wang, 1961).

Agricultural spray irrigation with wastewater is widely practiced throughout the world, and in many countries it is used indiscriminately without consideration of the pathogenic bacteria and viruses often present in domestic wastewater. One of the aspects of spray irrigation is the aerosolization of these entities and the possible spread of human disease by contaminated air (Katzenelson, 1976). Aerosolized enteric bacteria have been found in the vicinity of wastewater treatment plants at a distance of 1,200 m. When wastewater was used for spray irrigation, the sprinkler droplets also contained enteric bacteria (Adams, 1970). A study was conducted to obtain data about the number and types of enteric bacteria dispersed into the air during spray irrigation with wastewater.

Coliform bacteria were found in the air at a distance of 350 m downwind from the irrigation line. In one case, *Salmonella* was isolated 60 m from the source of irrigation (Katzenelson, 1976).

Armen and Ingham (2000) studied the decline of *E. coli* O157:H7 and *S.* Typhimurium DT104 in land-spread bovine manure exposed to different soils, temperatures, and moisture regimes. Their results demonstrated that bacterial death declined more rapidly at 25^{o}C than 10^{o}C; *E. coli* O157:H7 declined more rapidly than *S.* Typhimurium DT104 under similar conditions. Neither soil type nor watering regimes seemed to have appreciably affected the rate of decline. Mixing manure with soil hastened the rate of decline compared to top-spreading. Results suggested that the critical factors for the death of manure-borne pathogens in soil are temperature and application. Plowing manure into soil, to enhance competitive interactions with soil organisms, and warm weather application offer the best conditions for eliminating potential pathogens.

Wild and domestic animals, including mammals, birds, reptiles, and insects, are another source of pathogenic bacteria in agricultural environments. Salmonellae have been isolated from the intestinal tracts of most warm-blooded and many cold-blooded animals. Birds can be a particularly important contamination source because of their ability to transmit bacteria over substantial distances. Insects are another potential vector for transfer of fecal material from sites of sewage or animal manure accumulation to produce production areas (Geldreich et al. , 1964). There is some indication that pollinating insects can serve as a means of transmitting enteric bacteria to flowers. Once contaminated, developing fruit may internalize the bacteria eliminating their accessibility to surface decontamination steps. A significant portion of the resident populations of rodents, rabbits, and other commonly occurring mammals in agricultural production areas are likely to harbor enteric pathogens and are potential sources of fecal contamination, either through direct contamination of the field or via contamination of irrigation waters (Geldreich and Bordner, 1971). Allowing domestic animals access to orchards may

result in the contamination of fresh fruits and tree nuts, particularly if gathered after having fallen to the ground. Such practices may be linked to the presence of *Salmonella* and *E. coli* O157:H7 in apple cider and apple juice (CDC, 1975; Goverd et al. 1979; Besser et al. 1993). Flume water has been identified as a potential source of coliforms, *E. coli*, and *Salmonella* spp. contamination or cross contamination during apple cider production (Goverd, 1979).

Entry of pathogens into plants

Microbial contamination of plant tissue is largely associated with the surfaces of fruits and vegetables, and the inner tissue of sound vegetables and fruit are often considered sterile. However, numerous investigators have reported isolating low number of bacteria from internal tissues of apparently intact vegetables (Lund, 1992, Robbs et al, 1996). Samish et al. (1962) studied ten fruits and vegetables grown on different farms, and found that bacteria, mostly gram-negative motile rods, representatives of Pseudomonadaceae and the Enterobacteriaceae, can occur within normal, sound raw fruit tissues.

A study by Chang et al. (1999) demonstrated that flowers are a port of entry to pepper seeds for *Xanthomonas c. vesicatoria.* They reported that *X. c. vesicatoria* did not move more than 9 cm from the injection site 56 days after inoculation. Seeds were collected from fruits developed from flowers that had been inoculated. The contamination rates were from 13-17% with different strains. Ercolani and Casolari (1966) sprayed tomato blossoms with a suspension of X. c. *vesicatoria* containing 10^6cells/ml. One month later, after fruits set, typical signs of the disease were observed on the leaves whereas the fruit were outwardly healthy. The bacterium was isolated from the center of these symptomless fruits. Getz et al. (1983) observed that lesions on mature tomato fruit could develop from the trichomes in young fruit epidermis and would be infection sites for bacteria. Although no evidence of direct penetration of *E. coli* O157:H7 through the intact cuticle of pepper was observed, cells might be entrapped in natural lesions and microcracks on the uninjured surface (Han et al., 2000).

Factors affecting the occasional development of cavities in the center of fermenting cucmbers were investigated by Samish and Dimant (1957). Observations suggest that the existence of microorganisms within healthy, normal cucumber tissue. Cucumbers, excised before the flowers had opened, were always found to be sterile when plated in the same manner as fully-grown fruit. Bacteria developed readily on tissue of cucumbers harvested at a more advanced stage, i.e. after the flowers had wilted, which indicated that the bacteria might enter the cucumber through the blossom end, possibly at the time of abscission of the flower. A follow-up study showed that bacteria were found in the center of the cucumber as well as in areas located near the stem and blossom ends of the fruit. Populations near the stem end were lower than those in either the middle or the blossom end of the fruit. This suggests their point of entry. There was no correlation between size of cucumber and number of bacteria in the juice, which may possibly indicate that their activity is not increased during the growth process. Only after injury of the cells through plasmolysis does a very rapid growth of bacterial population take place (Samish and Dimant, 1959).

Meneley et al. (1974) found that the internal microflora of field-grown vegetables may provide a source of opportunistic bacterial pathogens as great as the microflora reported to be epiphytic; 44% of cucumbers in four samples, each comprising 25 fruits, contained bacteria. A mixture of bacteria, primarily representing the Enterobacteriaceae, Pseudomonadaceae, Corynebacteriaceae, Bacillaceae, and Micrococcaceae, were encountered. In a separate study using same approaches, they did not detect bacteria within tissues of trellised, sand-cultured, glasshouse-grown cucumbers obtained from a commercial operation. This corroborates the studies of Geldreich et al. (1964), who did not detect coliforms on indoor cultivated foliage, and Leben (1972) who found markedly fewer bacteria on the surfaces of greenhouse plants as compared to those grown in the field. It is certain that cultural and environmental conditions during growth of the fruit influences the presence and number of bacteria.

Meneley et al. (1975) injected a pathogenic isolate of *Erwinia carotovora* into the centers of cucumber fruits attached to the vine without causing disease. No macroscopic or microscopic symptoms were observed during the 5- to 8- day incubation period. The bacterium, however, was isolated from the internal tissues of the inoculated, harvested fruits. These bacteria can, perhaps, successfully penetrate the wall and membrane without disturbing the structure or physiology of the cell. Once penetration of the primary wall has been achieved, uptake could occur by endocytosis.

Samish et al. (1961) reported that bacteria are quite frequently present in the tissue of normal healthy tomatoes and occur equally in the green and red ripe fruits. The most common bacterial type found within the tomato is a motile rod belonging to the family Pseudomonadaceae. Low numbers of bacteria within tomatoes were associated with overhead irrigation. The bacteria did not appear to multiply and cause spoilage in the fruit. Growth was probably restrained by the pH, organic acids, and other inhibitors. Samish et al. (1963) postulated that variability of infestation in different lots of tomatoes could be due to varietal characteristics, climatic influences, or agrotechnical practices.

Samish et al. (1963) also studied the distribution of bacteria within healthy tomatoes. Among 222 tomatoes collected from different farms, 84 were sterile. Bacteria were detected in 62 % of the tomatoes, 35% of the stem-depression, and about 27% of the inner tomato pulp. In 12 fruits, bacteria were found within the pulp but not in the stem-depression. In 41 of 49 tomatoes, the bacterial species within the fruit were identical with those in the stem-depression. They also found that tomatoes from farms using overhead irrigation contained bacteria far more frequently and at higher populations than those from farms using irrigation in furrows. Possibly, overhead irrigation produces a microclimate more favorable to bacterial development.

Application of bacteria to the surface of fruits resulted in internalization over time (Samish et al., 1963). To study how bacteria might possibly enter into the fruits, two to three drops of *Serratia* suspension were applied on the outer side of the sepals of young Marmand tomatoes. The tomatoes were harvested within 1 to 5 weeks after treatment,

and the bacterial content was determined after surface disinfection. The stem-depression, which is exposed after the tomato stem is removed, was very often populated by bacteria, more so than the underlying fruit pulp. This suggests that bacteria may penetrate into the inner pulp from this area. Bacteria belonging to the same families as those found in the stem-depression are quite commonly found on the leaves of the tomato plant, on the sepals, and, at times, also on the surface of the tomato itself. Thus, it is possible that some bacteria find a suitable medium for survival and multiplication in the stem-depression, and penetrate into the growing fruit. This was supported by a study in which *Serritia* applied on the sepals of growing tomatoes were later recovered from stem-depression, as well as in the fruit pulp. The central core of the tomato had a higher bacterial population than the peripheral tissue. Representatives of the two bacterial families most often found within the tomatoes belong to the normal epiphytal microflora of these plants. Cells presumably progress into the fruit tissue more readily than cells of other members of the microflora because of their comparatively smaller size and motility.

Attachment and infiltration of foodborne pathogens to fruits and vegetables

Using scanning electron microscopy (SEM), Liao and Sapers (2000) observed that *Salmonella* Chester attached to apple stem and calyx cavity areas rather than intact skin. Firm attachment of cells on the calyx, stem, and injured tissue were resistant to sanitizer treatment. A large percentage (94%) of attached bacteria was found on stem and calyx cavity area. At pH 4.1, apple disks supported growth of *S.* Chester at 20^{o}C, but not at 8^{o}C. *E. coli* O157:H7 has been observed attach to the surface, trichomes, stomata, and cut edges of lettuce leaves (Seo, 1999).

Burnett et al. (2000) used confocal scanning laser microscopy (CSLM) to demonstrate the attachment of *E. coli* O157: H7 transformed with GFPuv plasmid to the surface and within the internal structures of nonwaxed apples. Results showed that infiltration through the floral tube and attachment to seeds, cartilaginous pericarp, and internal trichomes occurred in all apples examined, regardless of temperature differential during inoculation, and *E. coli* O157: H7 attached to internal core structures or within

tissues of apples may evade decontamination treatments. It was also observed that warm whole apples immersed in cold (2^{o}C) 1% peptone water containing 3×10^{7} CFU/ml, occasionally internalized the pathogen. The greatest extent of contamination on a per gram basis was consistently associated with outer core region, followed by skin and pulp (Buchanan, 1999). Dye uptake studies with Golden Delicious apples indicated that approximately 6% of the warm apples immersed in cold dye solution accumulated dye via open channels leading from the blossom end into the core region (Buchanan, 1999). Some uptake of the dye was also observed through the skin, particularly in regions where the apple had been bruised or the skin was punctured.

In apple cider production where the entire apple is pressed, pathogens found within the apple core are a potential problem. Internalization of *E. coli* in apples under natural environmental conditions was studied by Seeman et al. (2000) using a controlled outdoor setting. A surrogate *E. coli* species was applied to topsoil. Apples were placed randomly on the soil much like drop or windfall apples. It was noted whether the apple fell calyx up or down, or on its side. *E. coli* was found in the inner core and flesh samples of Yellow Delicious apples. Population ranged from 486 CFU/g and 1270 CFU/g in the inner core on days 1 and 8, respectively, and 538 CFU/g and 683 CFU/g in the flesh on days 1 and 8. Similar results were observed in the inner core and flesh of Red Delicious and Roma apples. Microorganisms located in openings are not as greatly affected by desiccation and other harsh environments, so that they are more likely to survive. When hydrocooling water becomes contaminated with spoilage bacteria, they subsequently contaminate the cooled products by entering through natural openings (lenticels, stomata) and mechanical injuries created during harvesting (Eckert, 1975).

A preliminary study to determine the bacterial infiltration potential of oranges was assessed by applying a suspension containing 10^{7} cfu of *E. coli* O157:H7 onto the stem scar, subjecting the oranges to a temperature decrease and juicing. Survival and growth studies were performed by injecting or applying pathogens to a simulated puncture wound located at various location on oranges, then incubating fruit for 5 days at either

4°C or 21°C. Results showed that oranges internalized *E. coli* O157:H7 at a frequency of 3.6%. Growth of *S.* Hartford and *E. coli* O157:H7 within oranges held at room temperature may result from compartmentalization in microenvironments within intact oranges. Segregated vesicles confine the acidic juice within oranges and, provided these vesicles are not disturbed, may create regions outside the vesicles where bacterial growth could occur (Walderhaug et al., 1999). In contrast, if these compartments are destroyed, e.g., during juicing operations, the acidic pH would prevail throughout the juice and provide a uniformly low pH environment for a microorganism. Their study supported the hypothesis that pH micro-environments do exist in the intact fruit and permit pathogen growth. Results also confirm previous reports demonstrating growth of human bacterial pathogens in internal fruit tissues of tomatoes, apples, and melons (Asplund, and Nurmi, 1991; Janisiewicz et al., 1999; Golden et al., 1993; Zhuang et al. 1995).

Hydroponically grown radish (*Raphanus sativus*) sprouts have been epidemiologically implicated as a vehicle for transmitting *E. coli* O157:H7 infection (WHO, 1996). Hara-kudo et al. (1997) studied the contamination of radish sprouts after exposure to *E. coli* O157:H7. The edible parts, the cotyledons and hypocotyl, became heavily contaminated with *E. coli* O157:H7 when sprouts were grown from seeds soaked in water contaminated with *E. coli* O157:H7. These same parts became contaminated after an 18-h exposure of the roots in *E. coli* O157:H7 cell suspension. These findings suggest that the presence of *E. coli* O157:H7 in the edible parts of radish sprouts could pose a serious hazard if the seeds or hydroponic water are contaminated with the bacterium. Mechanisms of spreading contamination, whether the bacteria spread through vessels, sieve tubes, or other parts of the hypocotyl, remain uncertain.

Gandhi et al. (2000) constructed GFP-expressing *E. coli* JM109 and *Salmonella* Stanley to investigate spatial location within alfalfa sprouts. Microscopy revealed marker bacteria at subsurface locations in association with root, hypocotyl, and cotyledon tissue. The study indicated that bacteria contaminating the outer surface of the alfalfa seed can localize at a subsurface level during sprout growth. Itoh et al. (1998) demonstrated the

presence of viable *E. coli* O157:H7 in inner tissues and stomata of cotyledons of radish sprouts grown in contaminated water.

Detection of *Salmonella* spp.

Contamination of fresh produce with *Salmonella* may occur at any point along the farm-to-table continuum, and probably at intermittent and low levels together with diverse naturally occurring microorganisms. Thus, rapid and sensitive methods for detecting *Salmonella* are critical to assure produce safety. It is not possible to test all lots of fresh-cut vegetables for all of the viral, bacterial, and parasitic pathogens that might be present. Even when sensitive methods are used, a large number of samples need to be analyzed from each lot to have a reasonable assurance of detecting contamination (http://www.dfst.csiro.au/fshbull/fshbull21.htm). The sporadic nature of most contamination makes it unlikely that microbiological testing will identify a lot of contaminated vegetables with any degree of certainty.

The International Commission on Microbiological Specifications for Foods (ICMSF, 1996) stated that "Good agricultural practices in growing crops, combined with acceptable hygienic methods during harvesting, packing, and transporting of vegetables are more important than microbiological testing, and routine microbiological examination of raw vegetables is unlikely to reduce hazards to any great extent and is not recommended. " However, appropriate microbial analysis is important in assessing the effectiveness of control program. Microbial analysis may also be used as a general measure of the hygienic conditions of growing, harvesting, transporting, and processing vegetables.

Standard cultural methods of detecting *Salmonella* in foods are laborious and time-consuming (3-7 days). Therefore, faster, reliable methods of detecting *Salmonella* are needed (Wolcott, 1991). Several rapid diagnostic assays for *Salmonella* can be placed in five general categories: miniaturized biochemical test; new media; instrumentation or automated systems; nucleic acid-based assays; and antibody-based assays (Tietjen, 1995).

Immunomagnetic separation (IMS) affords the advantage of relatively rapid antigenic captures (compared with ELISA). IMS coupled with an electrochemical method and electrochemiluminescent sensor were developed by Che (1999) and Yu (1996) respectively. These methods can detect *Salmonella* at populations as low as 10^3 CFU/g of food. Tan (1999) described an automated method combining IMS and selective liquid media to detect 2-25CFU/25g egg sample in 24h. Due to the presence of endogenous polyphenols, which are ubiquitous in plant products, IMS needs to be modified to facilitate rapid screening of fruit juices and plant produce for pathogens (Ogunjimi, 1999).

One of the most promising methods for detecting *Salmonella* is based on PCR, which combines simplicity with a potential for high specificity and sensitivity in detecting pathogenic bacteria in food. Several PCR assays specific for various *Salmonella* genes such as the *invA* gene (Rahn, 1992, Wang, 1995), the insertion sequence IS200 (Cane, 1993), the 16S rRNA gene (Iida, 1993), the *agfA* gene (Doran, 1993), the virulence-associated plasmid genes (Mahon, 1993, Rexach, 1994), and the *ViaB* sequence (Hashimoto, 1995) have been reported. A commercial BAX™ system has been evaluated in poultry and milk samples (Bailey, 1998; Bennett, 1998). A new fluorogenic TaqMan PCR assay has also been evaluated for detecting *Salmonella* in raw meat and shrimp (Chen, 1997; Kimura, 1999).

Invasion is an important factor influencing virulence of *Salmonella* spp.. The invasive phenotype is determined by a large cluster of genes present in *Salmonella* pathogenicity island 1 (SPI1) (Mills, 1995), which is present in all invasive strains of *Salmonella* (Galán, 1996). Among SPI1 virulence genes, *hilA* is a positive transcriptional regulator of several invasion genes (Bajaj, 1995). *SirA*, however, is a gene located outside the SPI1 (Johnston, 1996) and known as a global regulator of invasion as well as several other genes mediating enteropathogenesis (Ahmer, 1999).

ERIC PCR is based on eubacterial ERIC sequences. ERIC sequences are 126 bp long and appear to be restricted to transcribed regions of the genome, either in intergenic

regions of polycistronic operons or untranslated regions upstream or downstream of open reading frames (Hulton et al., 1991). ERIC sequences are highly conserved at the nucleotide sequences level but the chromosomal locations differ between species (Hulton et al., 1991).

These techniques have been used for rapid identification and characterization of *Actibacillus seminis* (Appuhamy et al., 1998), epidemiological studies of *Salmonella* spp. (Burr et al., 1998), and drug resistance analysis of *Enterobacter cloacae* (Zaher et al., 1997). They are also used in DNA typing of *Campylobacter jejuni* (Endtz *et al*., 1993), *Listeria monocytogenes* (Sciacchitano, 1998), and *E. coli* strains from cows with clinical mastitis (Lipman et al., 1995).

GFP as a marker for monitoring behavior of microorganism

Bacteria with bioluminescence markers can be used as positive control strains in laboratories and also to monitor cross contamination in food processing plants (Mead, 1994), microbial growth, survival, and colonization under various conditions (Dane, 1996, Fukui, 1996), and to test the effects of antibiotics, chemicals, and preservatives on the bacteria (Meighen, 1991). Chen (1996) has reported that *luxA* and *luxB* genes from *Vibrio harveyi* carried by a *Tn*5-containig plasmid could be introduced into *Salmonella* Enteritidis to monitor contamination and survival in eggs. Prachaiyo and McLandsborough (2000) introduced enhanced green fluorescent protein (EGFP) into *E. coli* JM 109 and *E. coli* O157: H7. The study showed that the expression of EGFP did not change growth kinetics or surface properties tested. Laser scanning confocal microscopy was used to observe interaction of the EGFP-expressing *E. coli* strains and the muscle components without changing spatial and temporal environment of the organisms.

GFP from the jellyfish *Aequorea victoria* is a reporter for monitoring transiently expressed genes. GFP emits bright green light upon exposure to UV or blue light and is unlike other bioluminescent reporters, which require additional proteins, substrates, or cofactors to emit light. GFP is a 27-KDa monomer consisting of 238 amino acids

(Prasher,1993). While intact GFP is required for fluorescence, the active chromophore in GFP is a cyclic tripeptide (Cody, 1993) whose sequence is present in the primary amino acid sequence. Chromophore formation is oxygen dependent, occurs gradually after translation (Inouye, 1994), and does not appear to be enzymatic (Heim, 1994).

GFP fluorescence is stable, species independent, and can be monitored noninvasively in living cells. The protein has a broad range of pH stability, retaining conformation from pH 5.5 to 12, and extreme thermostability, surviving temperatures up to 65^{o}C (Bokman, 1981). Fratamico et al. (1997) reported that *E. coli* O157:H7 expressing GFP was able to survive in orange juice at pH 3.51-3.59 for 24 day.

Most of the research to date with GFP in plants has been performed at the cellular level. Perhaps the most exciting applications will be at the whole plant level. For these studies, only modified versions of GFP have been used because of the requisite high expression. GFP has been used to monitor and study infectious plant pathogens (Laffel, 1997). Fluorescent protein as a replacement for potato virus X (PVX) protein coating has been used to study movement of virus through and among host plants (Oparka, 1996). Under illumination of UV light, viral movement can be easily monitored in whole plants.

Detection of GFP-expressing bacteria does not require problematic detection treatments such as the use of stains or immunofluorescence antibodies, which may interact with natural microbial flora. The GFP marker also facilitates easy and accurate enumeration of both injured and healthy cells without the use of selective media. For conventional scanning and transmission electron microscopy, fixation and dehydration techniques used in sample preparation can cause distortion of morphology and other artifacts (Little, 1991).

Tomato production

Tomatoes are of particular interest because they are consumed in large volumes, have been implicated in outbreaks of salmonellosis, are handled extensively in postharvest operations, and the general misconception that they do not support pathogen growth.

In the U.S., tomatoes are washed after harvest and before shipment. If the fruits are immersed in water that is cooler than the fruit a decrease in the internal gas pressure occurs. This results in an intake of water and bacteria through the stem scar (Bartz and Showalter 1981; Zhuang et al., 1995). The ability of foodborne pathogenic bacteria to grow on the cut surfaces of tomatoes and some other fruits has been of recent concern.

Tomato belongs to the genus *Lycopersicon*, with *L. esculentum* grown for edible fruit. There is increasing public interest in consuming foods that can have a significant effect on bodily health and that contain substantial levels of vitamins, minerals, and antioxidants. The tomato fruit has attracted considerable attention because of its substantial quantities of lycopene, an antioxidant, vitamin A, and ascorbic acid. The U.S. is one of the top five leading tomato-producing countries. However, the demand for tomatoes has increased substantially worldwide. In 1985, per capita consumption of fresh tomato fruit in the U.S. was 16.6 lb, increasing to 18.8 lb in 1995 (USDA, 1997). Consumption of tomatoes in the U.S. has reached 4.3 billion pounds each year. It is anticipated that per capita fresh fruit consumption will continue to increase due to the health benefits they provide.

The tomato is a herbaceous perennial, but is usually grown as an annual in temperate regions since it is killed by frost. It originally had an indeterminate plant habit, continuously producing three nodes between each inflorescence (Jones, 1998).
The majority of fresh market fruit is field grown. The principal advantages of hydroponic, controlled-environment agriculture, compared to field grown produce, is the isolation of the crop from the soil, which often introduces problems of diseases, pests, salinity, poor structure, and drainage. In general, greenhouse-grown fruit is vine ripened and can be delivered to the local market within a day or two of harvest. Most field-grown fruit is harvested before it is fully ripe and shipped to the market. Ripening occurs either naturally during shipment or as a result of ethylene treatment (Abeles et al., 1992). The calyx and stem are normally removed if the fruit is not packaged to minimize damaging other fruit, or the calyx is kept on the fruit to make the fruit look as if it has just been

taken from the plant. More than 90% of the fresh weight of tomato fruit is water, and the availability of water to the plant can influence fruit size. As tomato fruits develop, the percentage of fresh weight that is sucrose decreases while reducing sugars increase.

The five growth stages of tomato plants include seed germination and early growth until roots emerge, seedling growth until transplanted into growing media, vegetative growth until the first flower opens, early fruiting from the time first flower opens until first fruit is picked, and maturing of fruit, starting from time first fruit is picked until crop is terminated.

A considerable amount of high quality water is needed during tomato production. For those using a complete nutrient solution to supply plants with essential elements, failure to compensate for their presence can lead to elemental imbalances and stressed plants.

Modern tomato varieties often have more than five yellow petals and green sepals. The five anthers are joined around the pistil in *Lycopersicon*. Cultivated tomatoes are self-fertile, and their style length is similar to the anther length, a characteristic that favors self-pollination. There is a significant positive relationship between mean daily radiant light exposure (400 – 700 nm) and number of flowers reaching anthesis in the first inflorescence. Plant density is also a factor that can influence flower abortion and development. The optimum space per plant is 0.35-0.40 m^2; plants in double rows at 80-cm spacings with 1.2 m between the double rows is optimum for most varieties.

Sanitation in the greenhouse is essential. Suckers, immature, misshapen fruit, and senescent leaves must be immediately removed from the greenhouse to keep the greenhouse floor and growing area completely free of discarded plant materials. Plant material that is infested with insects or diseases must be immediately removed from the greenhouse and destroyed. Procedures for insect population control can be instituted by using chemical or biological treatments.

Research needed to reduce foodborne illnesses associated with raw produce

Salmonellae are one of the most frequently reported causes of foodborne illnesses in the U. S. Several multistate outbreaks of salmonellosis have been reported associated with raw produces. However, lack of information on sources of contamination of raw fruits and vegetables before and after harvest, location of contamination on or in the raw fruits, and sensitive analytical techniques makes it difficult to select appropriate intervention steps for reducing the risk of illnesses.

We hypothesize human pathogens, such as *Salmonella* spp. may possibly migrate into and survive in plant materials at some points during preharvest and postharvest stage. In order to study mechanism of contamination of fresh produce, tomatoes will be used as a model to determine conditions that influence migration, growth, survival and death of human pathogen *Salmonella*. A rapid and sensitive analytical method will be developed to detect low numbers of *Salmonella*, on or in raw fruits and vegetables.

The objectives will be as follows:

1. To design and evaluate a rapid and sensitive assay to detect *Salmonella* spp. present in tomatoes .
2. To investigate the establishment of *Salmonella* in tomato plants through different routes before harvest, such as root uptake, stem injection and flower brushing.
3. To evaluate water and soil as potential contamination sources of *Salmonella* during postharvest process.

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CHAPTER II

PCR DETECTION OF *SALMONELLA* MONTEVIDEO IN AND ON RAW TOMATOES USING PRIMERS DERIVED FROM *HILA*[1]

[1] Guo, X., J. Chen, L. R. Beuchat, and R. E. Brackett. Submitted to *Applied and Environmental Microbiology*.

ABSTRACT

Salmonella has been one of the most frequently reported etiological agents in fresh produce-associated outbreaks of human infections in recent years. PCR assays using four innovative pairs of primers derived from *hilA* and *sirA*, positive regulators of *Salmonella* invasive genes were developed to identify *Salmonella* Montevideo on and in tomatoes. Based on examination of eighty-three *Salmonella* and twenty-two non-*Salmonella* strains, it is concluded that a pair of *hilA* primers detects *Salmonella* specifically. The detection limits of the PCR assay were 10^1 and 10^0 CFU/ml after enrichment at 37°C for 6 and 9 h, respectively. When the assay was validated by detecting *S.* Montevideo in or on artificially inoculated tomatoes, 10^2 and 10^1 CFU/g were detected, respectively, with enrichment for 6 h at 37°C. Results suggest that the *hilA*-based PCR assay is sensitive and specific, and can be used for rapid detection of *Salmonella* in or on fresh produce.

INTRODUCTION

Salmonella is one of the most prevalent foodborne pathogens in United States. It is estimated to cause approximately 1.5 million cases of infection, with 15,000 hospitalizations and 500 deaths annually (26). Historically, salmonellosis has most often been associated with consumption of contaminated food of animal origin, such as poultry, eggs, meat, and dairy products. Changes in agronomic practices and dietary habits, and increased importation of fresh produce are thought to contribute to increased numbers of outbreaks associated with fruits and vegetables in recent years (3). Outbreaks of salmonellosis have been linked to tomatoes (12, 35), seed sprouts (24, 29, 33), watermelons (8, 10, 17), cantaloupes (11, 32), orange juice (14), and apple cider (9).

Consumption of fresh tomatoes was epidemiologically linked to 176 cases of *Salmonella* Javiana infections in Illinois, Michigan, Minnesota, and Wisconsin in 1990 (35). In 1993, tomatoes were identified as the vehicle for a multistate outbreak of *Salmonella* Montevideo infection (12). Zhuang *et al*. (36) described conditions influencing survival and growth of *Salmonella* Montevideo on the surface of intact tomatoes. Rapid growth occurred in chopped ripe tomatoes (pH 4.1±0.1) at ambient temperature. *S.* Enteritidis, *S.* Infantis, and *S.* Typhimurium were reported to grow in fresh cut tomatoes (pH 3.99 to 4.37) at 22 and 30^{o}C (4).

Contamination of fresh produce with *Salmonella* may occur at any point along the farm-to-table continuum, and probably at intermittent and low levels together with diverse natural flora. Thus, rapid and sensitive methods for detecting *Salmonella* are in great demand to assure produce safety. One of the most promising methods for detecting *Salmonella* is based on the polymerase chain reaction (PCR), which combines simplicity with specificity and sensitivity in detecting the pathogen in food. Several PCR assays have been developed by targeting various *Salmonella* genes, such as *invA* (30, 34), 16S rRNA (20), *agfA* (15), and *ViaB* (19), and virulence-associated plasmids (25, 31). These

PCR assays are mainly used for detecting *Salmonella* in poultry, meat, and milk samples (5, 7, 13, 23). Few have been applied to detect the pathogen in fresh produce.

Invasion is an important factor influencing virulence of *Salmonella* spp.. The invasive phenotype is determined by a large cluster of genes present in *Salmonella* pathogenicity island 1 (SPI1) (27), which is present in all invasive strains of *Salmonella* (16). Among SPI1 virulence genes, *hilA* is a positive transcriptional regulator of several invasion genes (6). *SirA*, however, is a gene located outside the SPI1 (22) and known as a global regulator of invasion as well as several other genes mediating enteropathogenesis (2).

In this study, PCR assays were developed using primers derived from *hilA* and *sirA*. The specificity and sensitivity of the assays were evaluated using pure cultures and further validated for detecting *Salmonella* in and on artificially contaminated tomatoes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Eighty-three *Salmonella* and twenty-two non-*Salmonella* (Table 2.1) strains used in this study were from the laboratory collections of Drs. Jinru Chen and Larry Beuchat at the Center for Food Safety and Quality Enhancement, University of Georgia. Before subjecting to PCR, strains were retrieved from frozen stock cultures and grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) at 37^{o}C for 16-18 h. Stock cultures were maintained on BHI agar at 4^{o}C throughout the project.

DNA preparation. Both crude and purified DNA were used in the PCR assays. Crude DNA was prepared by boiling bacterial cell suspensions. One milliliter of overnight bacterial culture was centrifuged at 12,000 $\times$ *g* for 2 min (Microcentrifuge 5415C, Eppendorf, Hamburg, Germany). Pellets were resuspended in 200 µl of sterile distilled water, boiled for 10 min, and centrifuged as described above. A 5-µl aliquot was

TABLE 2.1. Bacterial strains tested in the study

Salmonella strains			Non-*Salmonella* strains	No. of strains
Serogroup	Serotype	No. of strains		
B	S. Agona	***2***	*Aeromonas sobria*	1
	S. Brandenburg	2	*Escherichia coli* ATCC 10789	1
	S. Bredeney	2	*Escherichia coli* O157:H7	2
	S. Heidelberg	2	*Enterobacter aerogenes*	1
	S. Indiana	2	*Klebsiella pneumoniae*	1
	S. Reading	2	*Serratia marcescens*	1
	S. Saintpaul	2	*Shigella dysenteriae* non-type I	1
	S. Schwarzengrund	2	*Shigella sonnei*	3
	S. Typhimurium	4	*Staphylococcus aureus*	2
C	***S.*** Braenderup	2	*Proteus vulgaris*	1
	S. Choleraesuis	1	*Psudomonas fluorescens*	1
	S. Haardt	2	*Yersinia enterocolitica*	7
	S. Hadar	2		
	S. Hartford	1		
	S. Infantis	3		
	S. Kentucky	1		
	S. Mbandaka	2		
	S. Montevideo	2		
	S. Muenchen	1		
	S. Newport	2		
	S. Ohio	2		
	S. Oranienburg	2		
	S. Tennessee	2		
	S. Thompson	2		
D	*S.* Baildon	1		
	S. Berta	2		
	S. Dublin	1		
	S. Enteritidis	17		
	S. Panama	2		
	S. Sendai	1		
E	*S.* Anatum	3		
F	*S.* Rubislaw	1		
G	*S.* Cubana	1		
	S. Poona	1		
I	*S.* Gaminara	1		
J	*S.* Michigan	1		
N	*S.* Urbana	2		
R	*S.* Johannesburg	2		

used as a template for PCR. When purified DNA was prepared, DNeasy^{TM} tissue kit (Qiagen, Valencia, Calif.) was used according to manufacturer's instructions.

Specificity of PCR assays for *Salmonella*. Four oligonucleotide primers (Table 2.2) derived from *hilA* (GenBank accession no. U25352) and *sirA* (GenBank accession no. U67869) were designed in this study and synthesized by GIBCO BRL (Rockville, Md.). The specificity of all primers was tested using 83 *Salmonella* and 22 non-*Salmonella* strains.

The 50-µl PCR reaction mixture contained PCR buffer, dNTP (0.05 mM each), primers (0.25 µM each), *Taq* polymerase (1 U, Roche Diagnostics, Indianapolis, Ind.), and DNA template (5 µl, equivalent to approximately 10^7 CFU/ml). PCR reactions were performed in a DNA Thermalcycler 480 (Perkin Elmer, Norwalk, Conn.) using 1 cycle at 94^{o}C for 5 min, followed by 30 cycles of 94^{o}C for 2 min, 62^{o}C for 1 min, and 72^{o}C for 1 min, with a final extension at 72^{o}C for 10 min. The PCR amplicons were analyzed by gel electrophoresis on 1% agarose (GIBCO BRL, Rockville, Md.) gel in 1× TBE buffer (0.089M Tris-borate, 0.002M EDTA, pH 8.0). The gel was stained with ethidium bromide and visualized using the Gel Doc System 2000 (Bio-Rad Laboratories, Hercules, Calif.).

Limits of detection of HILA2-based PCR. *S.* Montevideo G4639 isolated from a patient in the 1993 tomato outbreak (36) was used for the sensitivity study and artificial inoculation of tomatoes. The organism was grown at 37^{o}C for 16-18 h in BHI broth with shaking in an incubator shaker (Model G-24, New Brunswick Scientific, Edison, N.J.). Cells were harvested when the culture was approximately at OD_{600} 1.0 (Spectrophotometer Novaspec II, Pharmacia Biotech, Cambridge, UK), plated on BHI agar, and incubated at 37^{o}C for 24 h before counting colonies. The cultures were serially diluted to give suspensions containing 10^0 to 10^9 CFU/ml in 10-fold increments. Crude DNA was prepared and amplified by PCR. Purified DNA from the *S.* Montevideo

TABLE 2.2. Oligonucleotide primers designed and used in this study

Primer name	Target gene	Sequence (5'-3')	PCR amplicon size
HILA1	*hilA*		
up		CGA CGC GGA AGT TAA CGA AG	972 bp
down		TCC TCC AAC TGA CCA GCC AT	
HILA2	*hilA*		
up		CTG CCG CAG TGT TAA GGA TA	497 bp
down		CTG TCG CCT TAA TCG CAT GT	
SIRA1	*sirA*		
up		AAG TTG TCG GTG AAG CGT GC	330 bp
down		TCT GAC TGA GCG CCA TCT GC	
SIRA2	*sirA*		
up		GCC GTA CTA ACG CCG TTG AC	430 bp
down		TAG CGA TAG CTG TTC ACC GT	

culture was diluted to an equivalent of 10^0 to 10^9 CFU/ml for PCR amplification. The effect of enrichment time on sensitivity was investigated. *Salmonella* cultures (10^9 CFU/ml) were serially diluted to give 10^0-10^8 CFU/ml. One-hundred µl was transferred to 900 µl of BHI broth, which was then incubated at 37°C with shaking for 3, 6, and 9 h. The cell cultures were harvested and amplified by PCR according to the above procedures.

PCR detection of artificially contaminated tomatoes. Raw, ripe Roma tomatoes with an average weight of 75 g were purchased from local grocery store, and divided into two groups for surface or internal inoculation. *S.* Montevideo was grown in BHI broth at 37°C for 16-18 h with shaking, and harvested when the OD_{600} reached 1.0, which corresponded to 10^9 CFU/ml. The culture was serially diluted in sterile 0.1% peptone water and 50 µl was used for inoculation. Negative controls consisted of sterile distilled water instead of cell suspension.

For surface contamination, each tomato was inoculated with 50 µl of *S.* Montevideo suspension. Populations in inocula ranged from 10^0 to 10^5 CFU per tomato. Inoculum was deposited in small drops to facilitate distribution and rapid drying. Inoculated tomatoes were then placed in a laminar flow hood and air dried for 2 h at 22 ± 1°C. Each tomato was placed in a stomacher bag with 20 ml of sterile 0.1% peptone water, and massaged by hand for 2 min. Rinse water was transferred into 50-ml Falcon centrifuge tubes, and centrifuged at 12,000 × *g* for 10 min (Centrifuge 5810R, Eppendorf, Hamburg, Germany). Pellets were resuspended in sterile 0.1% peptone water. Five milliliters of suspension were combined with 5 ml of BHI broth for enrichment. Cells were harvested after 6 h of enrichment and PCR was conducted. Plate counts were done using BHI and bismuth sulfite agar (BSA, Difco) to estimate the numbers of cells.

For internal inoculation, *S.* Montevideo was injected into tomato around stem scar area using a 20-µl pipette tip such that population was ca. 10^0, 10^1, 10^2, 10^3, 10^4 and 10^5

CFU/tomato. The inoculated tomato was placed into a filtered stomacher bag with 50 ml of sterile 0.1% peptone water, and stomached for 1 min at normal speed using a Stomacher 400 (Seward, London, UK). The liquid portion was transferred into a 50-ml Falcon centrifuge tube and centrifuged at $3{,}000 \times g$ for 5 min; the supernatant fluid was again centrifuged at $3{,}000 \times g$ for 5 min before transferring to a second tube and centrifuging at $10{,}000 \times g$ for 10 min. Plate counts and PCR were done as described above.

RESULTS

Specificity of the PCR assay for bacterial cultures. Among four pairs of primers (HILA1, HILA2, SIRA1, and SIRA2) evaluated, HILA2 detected only *Salmonella*. All 83 *Salmonella* strains tested positive, whereas non-*Salmonella* strains were not detected. Figure 2.1 shows representative PCR products from *Salmonella* and non-*Salmonella* strains using HILA2. The other three primers, HILA1, SIRA1, and SIRA2, generated non-specific bands (data not shown). HILA1 yielded non-specific bands with *Yersinia enterocolitica*, whereas SIRAl produced non-specific products from *Escherichia coli, Shigella sonnei, Y. enterocolitica,* and *Pseudomonas fluorescens.* The false positive results using SIRA2 were associated with *E. coli, Shigella sonnei, Klebsiella pneumoniae, Serratia marcescens,* and *Y. enterocolitica*.

Limits of detection of PCR assay for *Salmonella* cultures. Sensitivity of the HILA2-based PCR assay was investigated. Figure 2.2.A indicates that crude DNA from a suspension containing 10^5 CFU/ml yielded positive amplification, whereas pure DNA extracted with the Qiagen kit provided better sensitivity, with a detection limit of 10^4 CFU/ml (Figure 2.2.B). The detection limit was as low as 10^1 and 10^0 CFU/ml with 6- and 9-h enrichments, respectively (Figure 2.3).

FIGURE 2.1 Specificity of HILA2-based PCR assay for *Salmonella*. Gel electrophoresis of PCR products in 1% agarose in 1× TBE buffer. (**A**) *Salmonella* strains. Lanes 1 and 22, 100 bp DNA ladders (GIBCO BRL). Lanes 2 through 20 are PCR products amplified from *S.* Anatum, *S.* Baildon, *S.* Cubana, *S.* Enteritidis, *S.* Gaminara, *S.* Hartford, *S.* Heidelberg, *S.* Infantis, *S.* Michigan, *S.* Montevideo, *S.* Muenchen, *S.* Newport, *S.* Oranienburg, *S.* Panama, *S.* Poona, *S.* Saintpaul, *S.* Thompson, *S.* Typhimurium, and *S.* Typhimurium DT 104. Lane 21 is a negative control. (**B**) Non-*Salmonella* strains. Lanes 1 and 22 are 100 bp DNA ladders (GIBCO BRL). Lane 2 is *S.* Typhimurium. Lane 3 through 20 are the negative results obtained from *Aeromonas sobria*, *Escherichia coli* ATCC 10789, *Escherichia coli* O157:H7 (2), *Enterobacter aerogenes, Klebsiella pneumoniae, Serratia marcescens, Shigella dysenteriae* non-type I, *Shigella sonnei* (3), *Staphylococcus aureus* (2), *Proteus vulgaris, Psudomonas fluorescens*, and *Yersinia enterocolitica* (3). Lane 21 is a negative control.

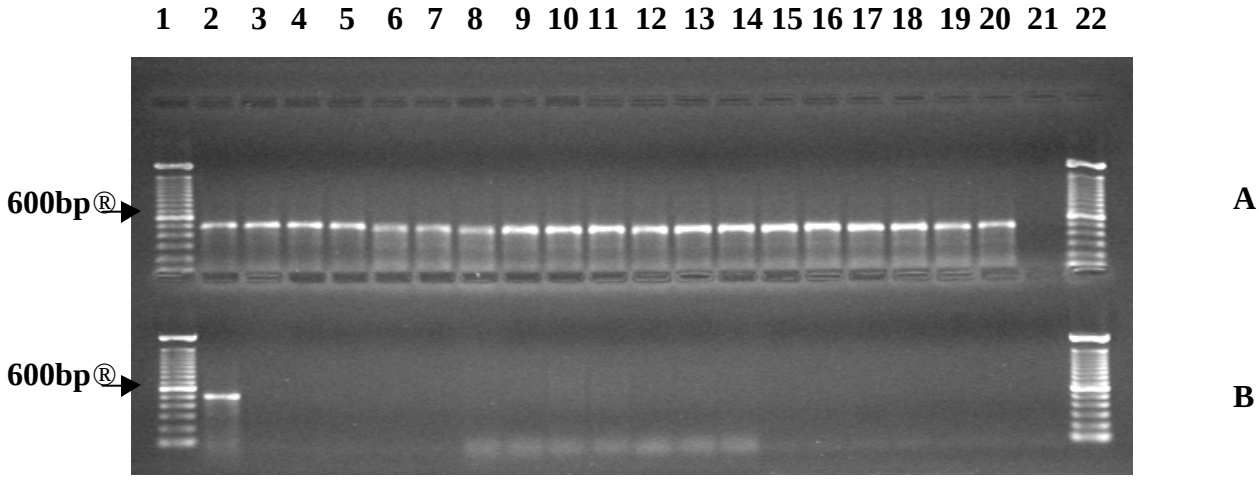
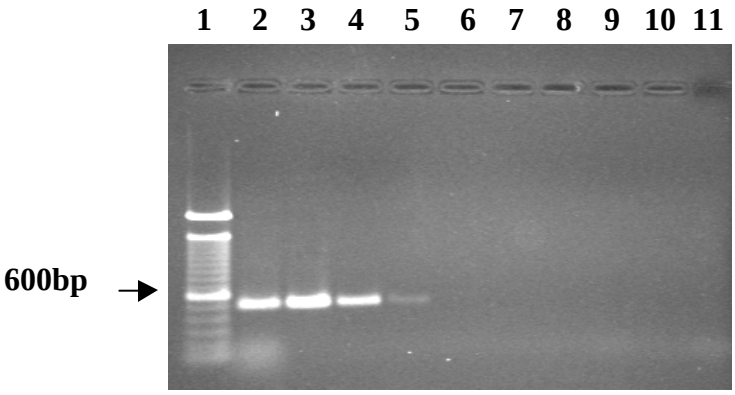
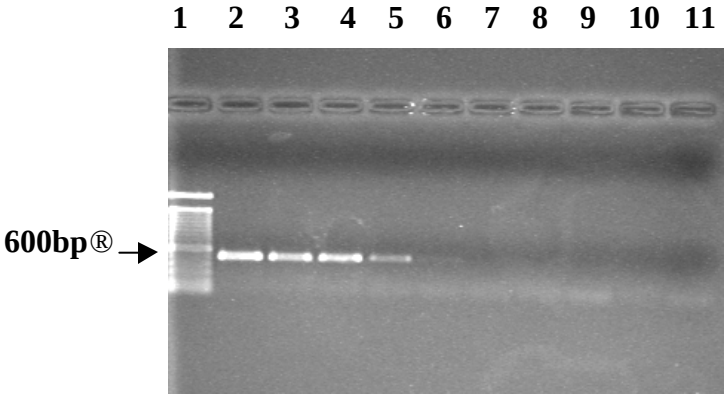

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
600bp®
600bp®
A
B

FIGURE 2.2 Limits of detection of HILA2-based PCR assay. (**A**) PCR products amplified from crude DNA. Lane 1 is 100 bp DNA ladders, lanes 2 through lane 10 are 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 10^0 CFU/ml. Lane 11 is a negative control. (**B**) PCR products amplified from purified DNA. Lane 1 is 100 bp DNA ladders, lanes 2 to lane 10 are DNA equivalent to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 10^0 CFU/ml. Lane 11 is a negative control.

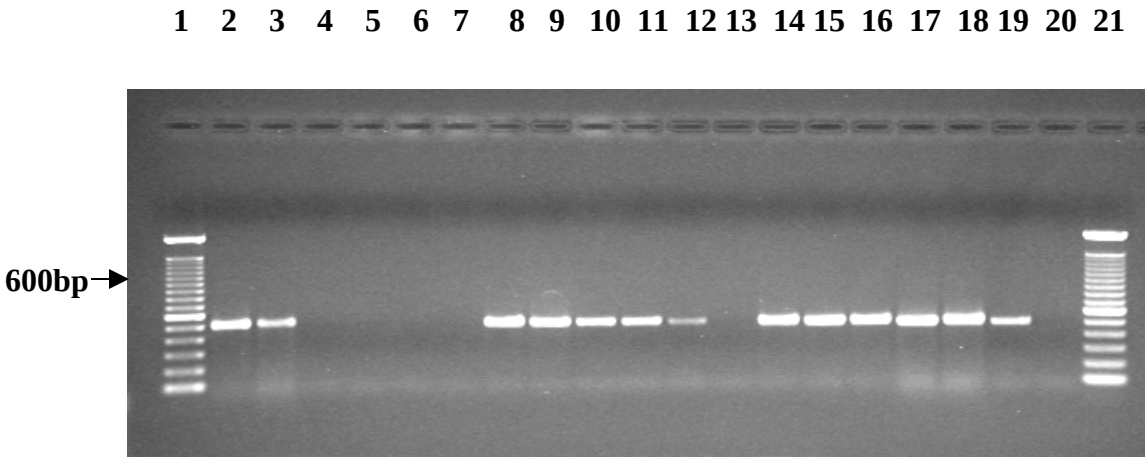


A



B

FIGURE 2.3 Limits of detection of HILA2-based PCR assay with different enrichment time. Cell cultures at 10^9 CFU/ml was serially diluted into 10^8-10^0 CFU/ml, and 100 µl of which was transferred to 900 µl BHI broth, which were then incubated at 37°C with shaking for 3, 6 and 9 h, respectively. The cells were treated and DNA amplified by PCR. Lanes 1 and 21 are 100 bp DNA ladders, lanes 2- 7, 8-13, 14-19 are PCR products from 10^5-10^0 CFU/ml after 3, 6 and 9-h enrichment, respectively. Lane 20 is a negative control.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
600bp

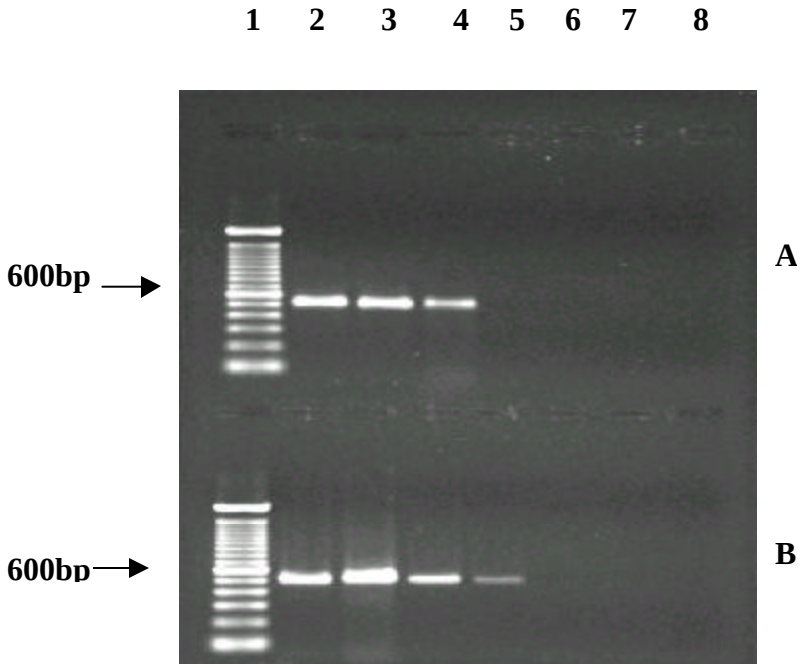
PCR detection of artificially contaminated tomatoes. *S.* Montevideo was recovered from inoculated tomatoes. Similar counts were obtained on BHI and BSA media. Without enrichment, the detection limit was 10^5 CFU/tomato on surface inoculated tomatoes (data not shown). When samples were enriched in BHI broth at 37°C for 6 h, detection limits were 10^2 and 10^3 CFU/tomato for *Salmonella* on and in tomatoes, respectively (Figure 2.4). Since the average weight of tomatoes was 75 g, the detection limits were equivalent to 10^1 and 10^2 CFU/g for *Salmonella* on and in tomatoes, respectively.

DISCUSSION

Various sets of primers for PCR detection of salmonellae have been reported (15, 19, 20, 25, 30, 31, 34). Gooding and Chaudary (18) recently conducted a comparison study of primers with different test panels of *Salmonella* and non-*Salmonella* strains and different PCR conditions and found that there were variations regarding specificity of primer. The results of our study indicate that the HILA2 primer set is highly specific to *Salmonella*, whereas HILA1, SIRA1, and SIRA2 primers generated false positive results for non-*Salmonella* strains.

The lack of specificity of SIRA primers may be explained by the roles of *sirA* genes in pathogenicity of *Salmonella*. It has been reported that all serovars of *Salmonella enterica* encode a type III protein secretion system within a pathogenicity island (SPI-1) at centisome 63 on their chromosome and this system is essential for their pathogenicity (16). HilA is a transcriptional regulator of the ompR/toxR family encoded within SPI-1 and controls expression of other SPI-1 genes (6). *SirA*, located outside the SPI-1, is an activator of *hilA* (22), also known as a global regulator of several other genes mediating enteropathogenesis (2). In addition, *SirA* appears to be a housekeeping regulator that has been adapted to virulence gene regulation in a variety of non-*Salmonella* bacteria, such as *Escherichia, Erwinia, Pseudomonas,* and *Vibrio* (1). *E. coli* possesses a *sirA* gene but

FIGURE 2.4 Use of HILA2-based PCR assay to detect *S.* Montevideo on and in artificially inoculated tomatoes. Tomatoes were spiked with different levels of *S.* Montevideo, cells were then recovered and enriched for 6 h. Crude DNA was prepared and subjected to PCR amplification. (**A**) Detection of *S.* Montevideo in tomatoes. (**B)** Detection of *S.* Montevideo on tomato surfaces. Lane 1 is 100 bp DNA ladders, lanes 2 to 7 are PCR products amplified from 10^5-10^0 CFU/tomato. Lane 8 is a negative control.


1 2 3 4 5 6 7 8
600bp
A
600bp
B

does not contain *hilA* or SPI-1 (2). Therefore, it was understandable that SIRA primer sets were not specific to *Salmonella*.

Although both crude and purified DNA worked well in PCR, the detection limits were somewhat different. Crude DNA obtained after boiling cells could be detected in suspensions containing 10^5 CFU/ml, whereas the threshold of extracted DNA increased 1 log in the suspension containing 10^4 CFU/ml. Due to a longer preparation time, DNA was prepared by boiling in further studies. If fresh produce is contaminated with *Salmonella*, low numbers of cells would be present, thus requiring enrichment for detection.

Tomatoes, which have been implicated as sources of *Salmonella* in several multistate outbreaks (12, 35), were chosen as a model in this study. The HILA2 primer set was validated to detect *S*. Montevideo on tomatoes. Fresh produce may contain a natural microflora that reflects production and harvest environments. Lactic acid bacteria, *Citrobacter, Enterobacter, Erwinia, Pseudomonas*, and *Flavobacterium* are frequently associated with plant products (21). Ogunjimi *et al*. (28) reported that endogenous polyphenol, which is ubiquitous in plant products, interfered with immuno-PCR detection of *E. coli* O157:H7. Moreover, ripe tomatoes have a pH range from 4.0 – 4.4. These factors may impact PCR detection of *Salmonella* in inoculated tomatoes. The results from this study showed that, in the presence of natural biota, *S*. Montevideo was recovered after centrifugation, and as low as 10^1 and 10^2 CFU/g could be detected on and in tomatoes, respectively, after 6 h of enrichment. The detection of *Salmonella* in samples that were subjected to internal inoculation was possibly affected by sample treatment. Lower populations of bacteria ($<10^2$ CFU/g) may not be accessible to PCR due to physical interference, and thus would not be detected by the procedure used in this study.

Results demonstrate that HILA2 is highly specific to *Salmonella*. The PCR assay, based on HILA2 primer, may also be applied for detecting *Salmonella* on other fresh produce.

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CHAPTER III

SURVIVAL OF *SALMONELLA* ON AND IN TOMATO PLANTS FROM THE TIME OF INOCULATION AT FLOWERING AND EARLY STAGES OF FRUIT DEVELOPMENT THROUGH FRUIT RIPENING[1]

[1] Guo, X., J. Chen, R. E. Brackett, and L. R. Beucthat. Submitted to *Applied and Environmental Microbiology*.

ABSTRACT

Salmonellae have been increasingly associated with outbreaks of infections associated with consumption of raw fruits and vegetables. Identification of mechanisms of contamination of raw produce in the field-to-table spectrum is essential before effective interventions to reduce the risk of illness can be devised. The fate of salmonellae applied to tomato plants was investigated. Five serotypes of *Salmonella* were used to inoculate tomato plants before and after fruits set, either by injecting stems or brushing flowers with inoculum. Ripe tomato fruits were subjected to microbiological analysis. Peptone wash water, homogenates of stem scar tissues, and homogenates of fruit pulp were serially diluted and plated on bismuth sulfite agar, before and after enrichment. Presumptive *Salmonella* colonies were confirmed by serological tests, PCR assay using HILA2 primers, and ERIC PCR. Results show that 43 and 40% of tomatoes from plants receiving stem inoculation before and after anthesis (flower opening), respectively, were positive for *Salmonella*; and 25% of tomatoes produced from flowers inoculated with *Salmonella* were positive for the pathogen. Higher percentages of surface (82%) and stem scar tissue (73%) samples, compared to pulp (55%) of tomato samples, harbored *Salmonella*. Among the five serotypes in the inoculum, *S.* Montevideo was the most persistent, being isolated from tomatoes 49 days after inoculation, and most dominant, being present in 4 of 11 (36%) *Salmonella*-positive tomatoes. Results suggest that *Salmonella* survives in or on tomato fruits from the time of inoculation at flowering through fruit ripening. Tomato stems and flowers are possible sites at which *Salmonella* may attach and remain viable during fruit development, thus serving as routes or reservoirs for contaminating ripened fruit.

INTRODUCTION

In recent years, outbreaks of infections associated with raw and minimally processed fruits and vegetables have occurred with increased frequency (19). Factors thought to influence this increase include changes in agronomic practices and dietary habits, and increased importation of fresh produce (2). *Salmonella* is estimated to cause approximately 1.5 million cases of foodborne infection each year in the U.S., with 15,000 hospitalizations and 500 deaths (27). Foods of animal origin, such as poultry, eggs, meat, and dairy products, have been historically recognized as vehicles of *Salmonella*. However, salmonellosis has been recently linked to tomatoes (13, 15, 37), seed sprouts (26, 29, 34), watermelons (6, 11, 21), cantaloupes (12, 30), and unpasteurized apple cider (10) and orange juice (14, 18).

In the farm-to-table production, processing, and distribution chain, there are various possible points of contamination of fruits and vegetables with disease-causing microorganisms. These include irrigation water, manure, wash water, handling by workers, and contact with contaminated surfaces (5, 32). It is essential that interventions be developed to prevent or minimize contamination of raw produce and to remove pathogens prior to consumption. To date, however, none of the chemical or physical treatments currently authorized by regulatory agencies for use to disinfect raw produce can be relied on to eliminate all types of pathogens from the surface or internal tissues (4). One of the keys to enable the selection of appropriate intervention steps to reduce populations of pathogenic microorganisms on fruits and vegetable is to identify sources of contamination, and to characterize the ecology of pathogens as affected by agronomic and processing practices (4, 8, 9).

Tomato is grown for its edible fruit. The U.S. is one of the top five leading tomato-producing countries. In 1985, per capita consumption of raw tomato fruit in the U.S. was 16.6 lb, increasing to 18.8 lb in 1995 (33). It is anticipated that the per capita consumption of raw tomatoes will continue to increase. Consumption of raw tomatoes has been epidemiologically linked to 176 cases of *Salmonella* Javiana infections in

Illinois, Michigan, Minnesota, and Wisconsin in 1990 (37). In 1993, tomatoes were identified as the vehicle for a multistate outbreak of *Salmonella* Montevideo infection (13). More recently, *Salmonella* Baildon was implicated in an outbreak with diced tomatoes in geographically separate areas of the U.S. (15).

Zhuang et al. (38) described conditions influencing survival and growth of *Salmonella* Montevideo on the surface of intact tomatoes. Rapid growth occurred in chopped ripe tomatoes (pH 4.1±0.1) at ambient temperature. *Salmonella* Enteritidis, *S.* Infantis, and *S.* Typhimurium were reported to grow in fresh cut tomatoes (pH 3.99 - 4.37) at 22 and 30^{o}C (3). Wei et al. (35) reported that *S.* Montevideo is able to multiply on wounded and cut tomatoes. The acidic pH (4.2 - 4.39 for ripe tomatoes and 4.33 - 4.52 for green tomatoes) did not completely inhibit growth. Weissinger et al. (36) reported that *S.* Baildon can grow in diced tomatoes (pH 4.40 ± 0.01); 0.79 $\log_{10}$ CFU/g increased to 5.32 and 7.00 $\log_{10}$ CFU/g within 24 h at 21 and 30^{o}C, respectively. However, after treatment with 200 µg/ml chlorine, diced tomatoes initially containing 0.60 - 0.86 $\log_{10}$ CFU of *S.* Baildon per g still harbored the pathogen (36). Chlorinated water is more effective in removing or inactivating S. Montevideo on tomato skin than in internal core tissue (35).

Ercolani and Casolari (20) demonstrated possible internalization of bacteria into tomato fruits by spraying tomato blossoms with a suspension of a plant pathogen, *Xanthomonas campestris* pv. vesicatoria. Typical symptoms of disease were observed on tomato leaves one month after inoculation. The bacterium was isolated from the center of fruits that did not show external symptoms of infection. A pathogenic isolate of *Erwinia carotovora* was injected into the center of healthy cucumber fruits attached to the vine without causing disease (28). However, the bacterium was detected from the internal tissues of fruits harvested from the inoculated plants. Samish et al. (31) studied ten fruits and vegetables grown on different farms, and found that bacteria, mostly gram-negative motile rods, representatives of Pseudomonadaceae and the Enterobacteriaceae, can occur within normal, sound raw fruit tissues.

The fate of human pathogenic bacteria applied to tomato flowers or inoculated into tomato stems before or after fruits set has not been described. We hypothesized that pathogens such as *Salmonella* may migrate through tomato stems and internalize tomato fruits. The possibility of internalization of *Salmonella* in tomato fruits developed from inoculated flowers was also considered. The objective of this study was to determine the fate of *Salmonella* inoculated into tomato stems and onto tomato flowers.

MATERIALS AND METHODS

Bacterial cultures. Five serotypes of *Salmonella* were used: *S*. Montevideo (serogroup C_1) was isolated from a patient in a tomato-associated outbreak, *S*. Michigan (serogroup J) was isolated from cantaloupe, *S*. Poona (serogroup G) was isolated from a patient in a cantaloupe-associated outbreak, *S*. Hartford (serogroup C_1) was isolated from a patient in an orange juice-associated outbreak, and *S*. Enteritidis (serogroup D) was from a patient in an egg-associated outbreak.

Inoculum preparation. Stock cultures maintained on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) at 4°C were transferred to BHI broth and incubated at 37°C. Cultures were transferred three times at 24-h intervals. Cells were harvested when OD_{600nm} reached ca. 1.0, which is equivalent to 10^9 CFU/ml. Each culture was centrifuged at $10,000 \times g$ for 5 min, washed with 0.01 M sodium phosphate buffer (pH 7.2) containing 0.85% sodium chloride (phosphate buffered saline, PBS) twice, and resuspended in 5 ml of PBS. Equal volumes of cell suspensions of each serotype were combined to form the inoculum for tomato plants.

Preparation of tomato plants for inoculation. 'Better Boy' tomato plants were used. Young, healthy plants ca. 20 cm in height were purchased from a local market. Plants were grown in a greenhouse (6.7 m × 7.3 m) equipped with ridge vents, an evaporative cooler, and a gas heater. The temperature was maintained at 25°C. Each plant was transferred to potting soil (Scotts Miracle-Gro Products, Port Washington, N.Y.) in a 3.8–liter plastic pot on June 6th, and placed in a polypropylene tray (Nalge

Company, Rochester, N.Y.) to retain water that filtered through the soil after applying water to the soil surface. Water was applied daily and Hoagland's nutrient solution (23) was applied weekly in volumes to maintain optimum soil moisture for plant growth, flowering, and fruit development.

Inoculation procedures. Tomato plants were inoculated with *Salmonella* when they started to bloom, which was 57 - 76 days after transplanting. One hundred open flowers on eight plants were gently brushed using a small paintbrush saturated with inoculum. Stems (1 - 2 cm diameter) were inoculated with cell suspension at a location ca. 5 cm from the flower base. Inoculum (50 µl) was deposited on the stem, which was then pricked with a #25 gauge syringe needle to facilitate contact with subsurface tissue. Stems were inoculated either before or after fruits set. Forty-nine stems were inoculated before fruiting and 41 stems were inoculated when fruits were 1 - 2 cm in diameter. Uninoculated plants served as controls. Both inoculated and control plants were grown in the same greenhouse.

Microbiological analysis. Tomatoes were harvested when subjectively judged to be 'red ripe' and ready for consumption. The weight of tomatoes ranged from 21 to 75 g. Each tomato was hand picked into a plastic zip-lock bag, sealed, and transported to the laboratory for analysis within 1 h. Tomatoes were immersed in 70% ethanol for 2 min to surface disinfect, then dried in a laminar flow hood at 22 ± 1^{o}C for 30 min. Each tomato was placed in a stomacher bag containing 20 ml of 0.1% sterile peptone water at 37^{o}C and hand rubbed for 2 min to dislodge surface population of *Salmonella* that may have evaded contact with ethanol. The peptone wash water was surface plated (0.25 ml in quadruplicate and 0.1 ml in duplicate) on bismuth sulfite agar (BSA, Difco). Plates were incubated at 37^{o}C for 24 h before examining for presumptive colonies of *Salmonella*. The stem scar tissue and pulp of each tomato were analyzed for the presence of *Salmonella*. Tomatoes were removed from the peptone wash water and the stem scar tissue was removed with a sterile scalpel. The stem scar tissue and remainder (pulp) of the tomato were separately placed in stomacher bags with 10 ml and 20 ml of sterile

0.1% peptone water, respectively, and pummeled at medium speed for 1 min with a Stomacher 400 (Seward, London, U. K.). Four 0.25-ml portions and two 0.1-ml portions of homogenate were surface plated on BSA. Plates were incubated at 37°C for 24 h before presumptive colonies were counted. Stem scar tissue and pulp homogenates were enriched by adding, respectively, 10 ml and 20 ml of universal preenrichment broth (Difco) and incubating the mixture at 37°C for 24 h. Cultures were streaked on BSA and incubated at 37°C for 24 h before examining before presumptive *Salmonella* colonies.

Colonies formed on BSA were examined for typical *Salmonella* appearance and morphology. Five presumptive colonies were randomly picked from each plate and transferred to BHI agar. Serological identification was performed using *Salmonella* antiserum groups C_1, D_1, G, and J (Difco) according to the manufacturer's instructions.

Confirmation of presumptive *Salmonella* colonies using PCR identification and DNA-based typing. PCR assays using HILA2 primer sets were done according to the procedure described by Guo et al. (22) to confirm presumptive isolates from inoculated tomatoes. PCR fingerprinting was also done to compare serotypes of isolates to those in the inoculum. The primer used for PCR fingerprinting was (5'-3') AAG TAA GTG ACT GGG GTG AGC G, based on a highly conserved, enterobacterial repetitive intergenic consensus (ERIC) sequence, which consists of 126 base pairs and appears to be restricted to transcribed regions of the genome, either in intergenic regions of polycistronic operons or in untranslated regions upstream or down stream of open reading frames (24). Crude DNA was prepared by boiling 20-h cultures of isolates in BHI broth for 10 min. One milliliter of culture was centrifuged at 12,000 $\times$ *g* for 2 min. Pellets were resuspended in 200 μl of sterile distilled water, boiled for 10 min, and centrifuged as described above. A 5-μl sample was used as a template for PCR. The 50-μl PCR reaction mixture contained PCR buffer, dNTP (0.4 mM each), primers (1μM), Taq polymerase (1U, Roche Diagnostics, Indianapolis, Ind.), and DNA template. PCR reactions were performed in a DNA thermal cycler 480 (Perkin Elmer, Norwalk, Conn.) using a cycle at 94°C for 5 min, followed by 40 cycles of 92°C for 45 sec, 25°C for 1 min,

and 68°C for 10 min, with a final extension at 72°C for 20 min. The PCR amplicons were analyzed by gel electrophoresis on 1% agarose (GIBCO BRL, Rockville, Md.) gel in TBE buffer (0.089 M Tris-borate, 0.002 M EDTA, pH 8.0). The gel was stained with ethidium bromide and visualized using a Gel Doc System 2000 (Bio-Rad Laboratories, Hercules, Calif.).

RESULTS

Approximately 80% of the inoculated flowers abscised from plants within 10 days after inoculation, regardless of the site or time of inoculation relative to fruit set. This may be due to lack of pollination, stress caused by inoculation, or other factors. Forty-three sound, red, ripe tomatoes were harvested and analyzed for presence of *Salmonella* (Table 3.1). Thirteen of these tomatoes were from plants not inoculated with *Salmonella*, and 30 were from plants inoculated either by stem injection or flower brushing. *Salmonella* was not detected on tomatoes from uninoculated control plants or by direct plating samples of peptone wash water, stem scar tissue homogenates, or pulp homogenates of tomatoes from control or inoculated plants. However, presumptive *Salmonella* was detected in enriched samples of peptone wash water, stem scar tissue, and pulp of tomatoes from inoculated plants. *Salmonella* was detected on or in tomatoes from plants receiving stem inoculation before or after flower set, and on or in tomatoes that developed from inoculated flowers (Table 3.1).

Eleven of thirty (37%) tomatoes harvested from inoculated plants were positive for *Salmonella*. Forty-three percent and 40% of the tomatoes receiving stem inoculation before and after flower set, respectively, were positive for *Salmonella*. Twenty-five percent of the tomatoes produced from inoculated flowers were positive for the pathogen. Compared to pulp of the tomatoes (6 of 11 positive, 55%), a higher percentage of the surface (9 of 11 positive, 82%) and stem scar tissue (8 of 11 positive, 73%) harbored *Salmonella*.

Of the eleven *Salmonella*-positive tomatoes, three contained the pathogen only on the surface, as evidenced by detection of the pathogen in peptone wash water but not

stem scar tissue or pulp (Table 3.2). *Salmonella* was detected in only stem scar tissue of two tomatoes, surface and stem scar tissue of one tomato, and all three sampling sites of five tomatoes, which indicates either systemic movement or cross contamination at the time of removal of fruits from the plants. In only one tomato (SA2), was more than one serotype detected. *S.* Hartford was not detected in or on tomatoes. Out of 11 *Salmonella*-positive tomatoes, *S.* Poona was present in five, *S.* Montevideo was present in four, and *S.* Enteriditis and *S.* Michigan were isolated in two.

All presumptive *Salmonella* isolates confirmed by PCR assay using the HILA2 primer set were subjected to PCR fingerprinting. The fingerprint results matched those of the serological tests. Fingerprint patterns of *Salmonella* isolates recovered from tomato samples matched the serotypes of *Salmonella* in the inoculum (Fig.ure 3.1).

A range of 21 - 49 days elapsed between the date of inoculation and sampling (Table 3.1). Fingerprint patterns and dates of isolation indicate that *S.* Montevideo was most persistent, being isolated 49 days after inoculation, and most dominant, being present on or in 4 of 11 (36%) tomatoes. *S.* Poona and *S.* Michigan were detected on and in tomatoes 40 and 39 days post-inoculation, respectively. *S.* Enteritidis was not detected in or on tomatoes harvested more than 27 days after plants were inoculated.

DISCUSSION

Tomato flowers feature style and anthers of similar heights, a characteristic that favors pollination. Tomato plants normally need bees or shaking of the plants for good pollination. In our study, most of the inoculated flowers aborted from the plant within 10 days. Chang et al. (16) reported that pepper flowers brush-inoculated with *X. c.* vesicatoria severed from plants 2 - 5 days after inoculation, and were a port of entry to pepper seeds for *X. c.* vesicatoria. This bacterium did not move more than 9 cm from the injection site on the stem 56 days after inoculation, and only seeds collected from fruits produced from inoculated flowers contained the bacterium.

TABLE 3.1 *Salmonella* on or in tomato fruits produced on control and inoculated plants.

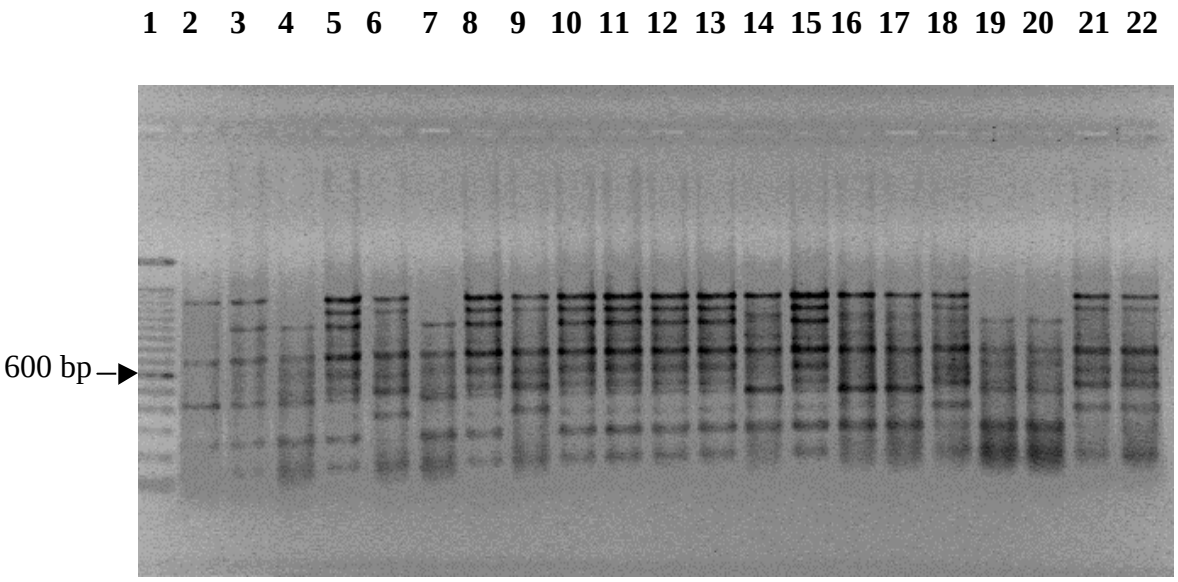
Inoculation Site	Time between inoculation and analysis (days)	Fruit				Presence of *Salmonella*[a]		
		Weight (g)	Number		Percent postitive	Peptone wash water	Stem scar tissue	Pulp
			Examined	Positive				
Control (not inoculated)		45 ± 24	13	0	0	0	0	0
Stem								
Before fruit set	27-49	55 ± 20	7	3	43	2	2	1
After fruit set	21-28	44 ± 19	15	6	40	5	5	4
Flower	27-45	54 ± 19	8	2	25	2	1	1

[a]Number of enriched samples confirmed to contain *Salmonella*.

TABLE 3.2. Serotypes of *Salmonella* isolated from tomato fruits.

Inoculation site and fruit number	Time between inoculation and analysis (days)	Serotype detected		
		Peptone wash water	Stem scar tissue	Pulp
Stem				
Before fruit set				
SB4	27	Poona	Poona	Poona
SB7	49		Montevideo	
SB13	40	Poona		
After fruit set				
SA1	28	Poona		
SA2	28	Poona	Montevideo	
SA5	28	Poona	Poona	Poona
SA8	27	Michigan	Michigan	Michigan
SA29	28		Montevideo	
SA32	22	Enteritidis	Enteritidis	Enteritidis
Flower				
F14	39	Michigan	Michigan	Michigan
		Montevideo	Montevideo	Montevideo
F30	27	Enteritidis		

FIGURE 3.1. DNA-based typing of presumptive *Salmonella* colonies isolated from tomato fruits. Lane 1, 100-bp DNA marker (GIBCO BRL); lanes 2 through 6, DNA profiles of *S.* Enteriditis, *S.* Hartford, *S.* Michigan, *S.* Poona, and *S.* Montevideo, respectively; lanes 7 through 22, DNA profiles of stem scar tissue of tomato SA8, stem scar tissue of SA5, pulp of SA29, wash water from SB13, wash water from SB4, stem scar tissue of SB4, pulp of SB4, wash water from SA32, wash water from SA1, pulp of SA32, wash water from F30, wash water from F14, wash water from F14, stem scar tissue of F14, pulp of F14, and pulp of F14. See Table 2 for key to tomato fruit numbers.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
600 bp

Observations on survival of *Salmonella* on or in tomato fruits (Table 3.1) indicate that the pathogen persisted on and in tomato plants from the time of inoculation at flowering through fruit ripening. Citric acid is the predominant acid in tomato fruit and the pH of pulp of most cultivars is below 4.5. More than 90% of the weight of tomato fruits is water. As tomato fruits develop, the amount of sucrose decreases while starch and reducing sugars increase (25), which would favor nutrient availability for growth of *Salmonella*. Growth of *S.* Anatum, *S.* Senftenberg, and *S.* Tennessee at pH 4.05 under otherwise ideal conditions has been reported (17).

The presence of *Salmonella* in peptone wash water, stem scar tissue, and pulp of tomatoes from inoculated plants indicates that flowers and injured stems could be possible routes of bacterial contamination of tomato fruits at different points during development and maturation. During plant growth, phytopathogens can penetrate the plant surface through natural openings such as stomata or leaf hydathodes, or through wounds (1). Some bacteria enter blossoms through the nectarthodes or nectaries, which are similar to hydathodes. However, bacteria enter plants most often through wounds, and less frequently through natural openings (1). Plant pathogens may grow briefly on or in wounded tissue before advancing into healthy tissue. Injection of *Salmonella* into the tomato stem may introduce the pathogen into xylem, which has the principal role of transporting water and nutrients from the root to the extremities of the plant. Additionally, in the secondary xylem, the axial and ray parenchyma store nutrients and water (7) which sustain viability of plants and, possibly, promote survival of human pathogenic bacteria. The presence of epiphytal flora within tissue of fruits and vegetables through various pathways was reported by Samish et al. (31). By examining eight internal locations of tomatoes, they observed that bacteria are unevenly distributed in the fruit, and entry may be from the stem scar tissue through the core and into the endocarp. This study suggested that some epiphytal flora might reach internal tissue of tomatoes, because of their small size and motility, through natural apertures. It may be that bacteria enter fruit tissue more readily in the early stages of fruit development, at a time when

various channels are not yet covered by corky or waxy materials (31). Broken trichomes on young fruits represent another site of entry of microorganisms.

Although *Salmonella* is a human pathogen, our study reveals its ability to survive on or in tomato fruits throughout the course of plant growth, flowering, and fruit development and maturation. Tomato stems and fruits are subject to mechanical injury in the field and during post-harvest handling, which make them more susceptible to internalization of bacteria. Irrigation water, manure, soil, water used to prepare fungicides and insecticides, and human handling are potential sources of *Salmonella*. Interventions need to be applied to eliminate contamination of tomato fruits with *Salmonella* by preventing or minimizing its contact with tomato plants and fruits at all points from the farm to the consumer.

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CHAPTER IV

SURVIVAL OF *SALMONELLA* ON TOMATOES STORED AT HIGH RELATIVE HUMIDITY, IN SOIL, AND ON TOMATOES IN CONTACT WITH SOIL[1]

[1] Guo, X., J.Chen, R. E. Brackett, and L. R. Beuchat. Submitted to *Journal of Food Protection*.

ABSTRACT

Salmonellosis has been linked to the consumption of several types of raw fruits and vegetables**.** The objectives of this study were to investigate water and soil as reservoirs of *Salmonella* for contamination of tomato fruits and survival of *Salmonella* in water and soil. *Salmonella* survived for at least 45 days in inoculated moist soil. The population of *Salmonella* on tomatoes in contact with soil increased by 2.5 $\log_{10}$ CFU/tomato during storage for 4 days at 20^{o}C, and remained constant for an additional 10 days. The pathogen on tomatoes spot-inoculated with a water suspension of cells decreased by ca. 4 $\log_{10}$ CFU/tomato during storage for 14 days at 20^{o}C and 70% relative humidity. Imaging of cut tomatoes revealed that, on fruits in contact with inoculated soil at 20^{o}C for 1 day, cells appeared only near or on the surface. More cells were observed in stem scar and subsurface areas of tomatoes as the time of storage increased. PCR fingerprinting revealed that among five *Salmonella* serotypes tested, *S.* Montevideo was the most persistent serotype detected on tomatoes stored on the surface of soil and on spot-inoculated tomatoes, followed by *S.* Poona and *S.* Michigan. Results also demonstrated that an EGFP marker can be used to detect and monitor growth of *Salmonella* in the presence of background microflora. Our observations on infiltration of *Salmonella* into tomatoes support the contention that preharvest contact of produce with contaminated water or soil exacerbates problems associated with its postharvest removal or inactivation.

INTRODUCTION

Salmonella is estimated to cause approximately 1.5 million cases of infection, with 15,000 hospitalizations and 500 deaths in the U.S. annually (24). Foods of animal origin such as poultry, eggs, meat, and dairy products have been historically recognized as potential vehicles of *Salmonella*. However, salmonellosis has also been linked to the consumption of seed sprouts (23, 26, 34), watermelons (5, 9, 19), cantaloupes (10, 29), unpasteurized apple cider (8), and unpasteurized orange juice (12, 14). Consumption of raw tomatoes was epidemiologically linked to 176 cases of *Salmonella* Javiana infections in Illinois, Michigan, Minnesota, and Wisconsin in 1990 (37). In 1993, tomatoes were identified as the vehicle in a multistate outbreak of *Salmonella* Montevideo infection (11). More recently, *Salmonella* Baildon was implicated in an outbreak linked to diced tomatoes in geographically separate areas of the U.S. (13).

In the farm-to-table production, processing, and distribution chain, there are various possible sources of pathogens and points of contamination of fruits and vegetables with disease-causing microorganisms. These include irrigation water, runoff water from adjacent livestock farms, manure, wash water, handling by workers, and contact with contaminated surfaces, animal fertilizers applied in previous growing seasons, or feces from rodents or ruminants (4, 33). It is essential that interventions be developed to prevent or minimize contamination of raw produce and to remove pathogens prior to consumption. One of the keys to enable the selection of appropriate intervention steps to reduce populations of pathogenic microorganisms on fruits and vegetable is to identify and eliminate sources of contamination, and to characterize the ecology of pathogens as affected by agronomic and processing practices (2, 6, 7).

Salmonella is occasionally isolated from horticultural crops and from water after washing raw fruits and vegetables (36). In Italy, Ercolani (16) detected *Salmonella* in wash water from 68% of the lettuce and 72% of the fennel samples tested. Rude et al. (30) cultured *Salmonella* from 4 of 50 vegetables examined in the U.S. In Spain, Garcia-Villanova-Ruiz et al. (18) cultured *Salmonella* in 7.5% of the market vegetables

examined. The U.S. Food and Drug Administration examined imported cantaloupes and watermelons in 1990 and 1991 and isolated several serotypes of *Salmonella* from approximately 1% of the rinds (10). As melons develop, they are in contact with the ground and may be contaminated with soil and animal feces that harbor *Salmonella*. Irrigation water may also be a source of contamination. The use of irrigation water containing enteric bacteria, viruses, protozoa, or helmiths results in increased frequency of isolation of pathogens from harvested produce (15, 25).

Green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*, is a 27-KDa monomer consisting of 238 amino acids (28) that emits bright green light upon exposure to UV or blue light. Bacteria with a fluorescence marker such as GFP can be used to facilitate monitoring survival, growth, and colonization under various ecological conditions. *Escherichia coli* O157:H7 expressing GFP, for example, was reported to survive in orange juice with pH 3.51 - 3.59 for 24 days (17). Prachaiyo and McLandsborough (27) introduced enhanced green fluorescent protein (EGFP) into *E. coli* to study interaction of the bacterium with muscle components.

We hypothesized that human pathogens such as *Salmonella* may attach to or infiltrate plant tissues at numerous stages of preharvest development and during postharvest handling. The objectives of this study were to investigate water and soil as reservoirs of *Salmonella* for contamination of tomato fruits. Survival of *Salmonella* tagged with EGFP in water and soil was also investigated.

MATERIALS AND METHODS

Bacterial cultures. Five serotypes of *Salmonella* were used: *S.* Montevideo (serogroup C_1) was isolated from a patient in a tomato-associated outbreak, *S.* Michigan (serogroup J) was from a cantaloupe, *S.* Poona (serogroup G) was from a patient in a cantaloupe-associated outbreak, *S.* Hartford (serogroup C_1) was from a patient in an orange juice-associated outbreak, and *S.* Enteritidis (serogroup D) was from a patient in an egg-associated outbreak. Transformation of the five *Salmonella* serotypes with an

EGFP (enhanced green fluorescent protein) plasmid (Clonetech Laboratories, Inc., Palo Alto, Calif.) was achieved using BioRad Gene Pulser® electroporator (BioRad Laboratories, Hercules, Calif.) using 2.4 kV, a field strength of 12.00kV/cm, capacitance of 25 μF, resistance of 400 Ω, and time constant of 9.7 msec. The transformants were transferred to brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) supplemented with 100 μg/ml ampicillin (Sigma, St. Louis, Mo.) (BHI/Amp agar), incubated at 37°C for 16-18 h, and examined under UV light (365 nm wavelength). Brightly green fluorescent colonies were picked and designated as *Salmonella*–EGFP.

Inoculum preparation. Stock cultures of five serotypes of *Salmonella* labeled with EGFP were maintained at –80°C. *Salmonella* serotypes were individually prepared by transferring stock cultures to 50 ml of BHI broth (Difco) supplemented with 100 μg/ml ampicillin (BHI/Amp broth) and incubated at 37°C. Cultures were loop transferred three times at 24-h intervals. Cells were harvested when the A_{600nm} of the broth reached ca. 1.0, which correlates to 10^9 CFU/ml. Each culture was centrifuged at 10,000 × *g* for 5 min, washed in 50 ml of 0.01 M sodium phosphate buffer (pH 7.2) containing 0.85% sodium chloride (phosphate buffered saline, PBS) twice, and resuspended in 50 ml of sterile tap water. Equal volumes of cell suspension of each serotype were combined to form an inoculum used in all experiments.

Inoculation procedures. Lightly waxed mature green tomatoes weighing 150 - 250 g each were purchased from local grocery store and divided into two groups. The first group was used to determine survival characteristics of *Salmonella* inoculated on the surface of tomatoes, following storage at 20°C. Cell inoculum (50 μl) was deposited in several small drops within a 3-cm diameter circle near the blossom end of tomatoes, followed by drying in a laminar flow hood for 2 h at 22 ± 1°C. Negative controls consisted of tomatoes on which 50 μl of sterile tap water instead of a cell suspension were applied. The inoculated tomatoes were placed in polypropylene trays (Nalge Company, Rochester, N. Y.), covered with aluminum foil, and stored at 20 ± 1°C.

The second group of tomatoes was used to study attachment and infiltration of *Salmonella* into tomatoes placed on the surface of water-saturated soil inoculated with the pathogen. Topsoil (Scotts Miracle-Gro Products, Port Washington, N. Y.) (2,500 g) moistened with 1000 ml of sterile tap water and 250 ml of inoculum containing 9.02 $\log_{10}$ CFU *Salmonella*/ml was spread in a 5-cm layer in polypropylene trays. Tomatoes were placed on the inoculated soil with the stem scar facing downward and firmly pressed to assure good contact with soil. Negative controls were placed on sterile, moistened topsoil. The tomatoes were then covered with foil and stored at 20 ± 1°C. All experiments were repeated three times.

Microbiological analysis. Tomatoes from each treatment were analyzed after storage for 0, 1, 2, 4, 7, 10, and 14 days. Visible soil was removed from the surface of tomatoes that had been incubated on top of the inoculated soil by rinsing with 100 ml of sterile 0.1% peptone water. Tomatoes were then placed in a stomacher bag with 20 ml of 0.1% sterile peptone water and hand massaged for 2 min. The wash fluid was serially diluted, and plated (0.1 ml in duplicate) on BHI/Amp agar and bismuth sulfite agar (Difco) (BSA). Plates were incubated at 37°C for 24 h before presumptive colonies were counted.

Washed tomatoes were surface disinfected by immersing in 100 ml of 70% ethanol for 5 min, then dried in a laminar flow hood at 22 ± 1°C for 30 min before analyzing for the presence of *Salmonella* at subsurface locations. Each tomato was cut in half with a sterile knife starting from blossom end and cutting toward the stem scar tissue. Tomato halves were placed cut-side down on BSA and BHI/Amp agar plates for 1 min, then removed and discarded. Plates were incubated at 37°C for 24 h. Colonies formed on BSA were examined for morphology, while those formed on BHI/Amp agar were observed under a UV light (365 nm) for fluorescence.

Tomatoes spot-inoculated but not placed on soil were analyzed for populations of *Salmonella* as described above for tomatoes stored on the surface of inoculated soil,

except that rinsing was not needed to remove soil. Tomatoes were discarded after washing in 20 ml of sterile 0.1% peptone water.

Presumptive *Salmonella* isolates from various samples were subjected to serological tests and ERIC PCR to determine if one or more serotypes in the inoculum were present or dominant on stored tomatoes and in inoculated soil. Five presumptive colonies were randomly picked from each plate containing 25 – 250 colonies, transferred to BHI agar, and incubated 24 h at 37°C. Serological identification of isolates was performed using *Salmonella* antiserum groups C_1, D_1, G, and J (Difco) according to the manufacturer's instructions. Distribution of *Salmonella* within cut tissues of tomatoes was observed using a Kodak Image Station 440CF (Eastman Kodak Company, Rochester, N.Y.).

Confirmation of presumptive *Salmonella* colonies using DNA-based typing. PCR fingerprinting was done to compare serotypes of isolates from tomatoes with those in the inoculum. The primer used for PCR fingerprinting was (5'-3') AAG TAA GTG ACT GGG GTG AGC G, based on a highly conserved, enterobacterial repetitive intergenic consensus (ERIC) sequence, which consists of 126 base pairs and appears to be restricted to transcribed regions of the genome, either in intergenic regions of polycistronic operons or in untranslated regions upstream or down stream of open reading frames (21). Crude DNA was prepared by boiling 20-h cultures of isolates in BHI broth for 10 min. One milliliter of culture was centrifuged at 12,000 × *g* for 2 min. Pellets were resuspended in 200 µl of sterile distilled water, boiled for 10 min, and the suspension was centrifuged as described above. A 5-µl sample was used as a template for PCR. The 50-µl PCR reaction mixture contained PCR buffer and dNTP (0.4 mM each), primers (1µM), Taq polymerase (1U, Roche Diagnostics, Indianapolis, Ind.), and DNA template. PCR reactions were performed in a DNA thermal cycler 480 (Perkin Elmer, Norwalk, Conn.) using a cycle at 94°C for 5 min, followed by 40 cycles of 92°C for 45 sec, 25°C for 1 min, and 68°C for 10 min, with a final extension at 72°C for 20 min. The PCR amplicons were analyzed by gel electrophoresis on 1% agarose gel

(GIBCO BRL, Rockville, Md.) in TBE buffer (0.089 M Tris-borate, 0.002 M EDTA, pH 8.0). The gel was stained with ethidium bromide and visualized using a Gel Doc System 2000 (Bio-Rad Laboratories, Hercules, Calif.).

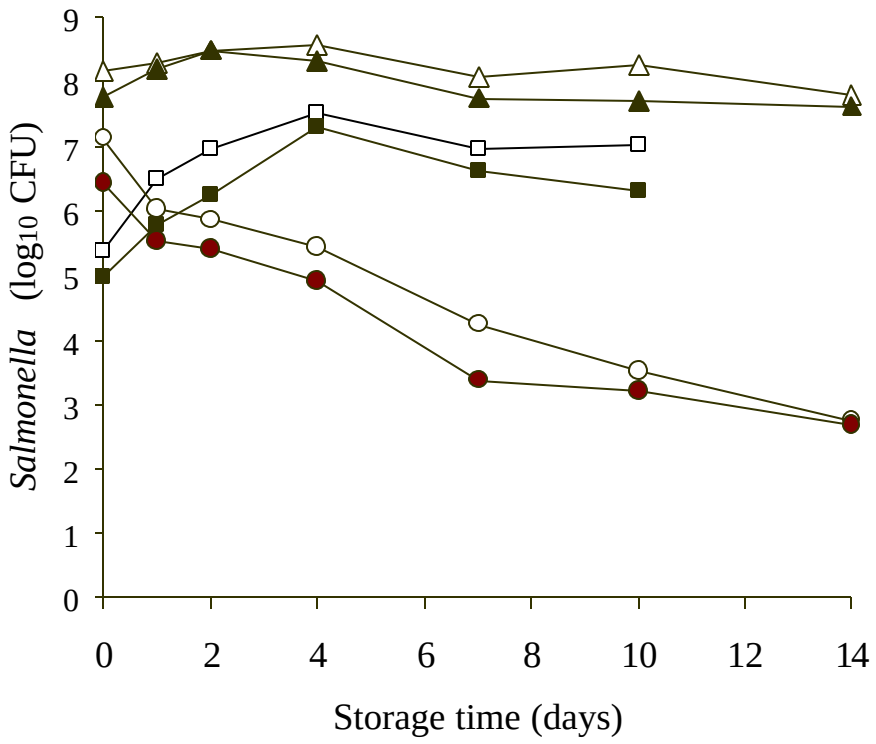
Statistical analysis. The Student t-test using Statistical Analysis Software (SAS Institute, Cary, N.C.) was used to determine significant differences ($P \leq 0.05$) in populations of *Salmonella* recovered on BSA and BHI/Amp agar from inoculated soil, spot-inoculated tomatoes, and tomatoes in contact with inoculated soil. Three replicate samples were analyzed.

RESULTS

Survival of *Salmonella* spot-inoculated on tomatoes. Each tomato was inoculated with 7.72 $\log_{10}$ CFU of *Salmonella*. The populations recovered on BSA and BHI/Amp agar on the day of after inoculation (day 0) were 7.10 and 6.42 $\log_{10}$ CFU/tomato, respectively. After storage of tomatoes at 20^{o}C in an environment with 70 ± 2% relative humidity for 1 day, ca. 1 log decrease in CFU/tomato occurred (Figure 4.1). The population gradually decreased by an additional 3 logs between 1 and 14 days. Higher populations were consistently detected on BSA compared to apparent populations on BHI/Amp agar. However, lost of the EGFP plasmid in a portion of the cells would result in non-fluorescent colonies on BHI/Amp agar. Since only fluorescent colonies on BHI/Amp agars were counted, populations of *Salmonella* were likely underestimated.

***Salmonella* on tomatoes in contact with soil.** After removal of visible soil residue from tomatoes stored on the surface of soil inoculated with *Salmonella* for up to 14 days, populations of the pathogen remaining on the tomato were determined. Population detected on BSA and BHI/Amp agar were 5.37 and 4.97 log CFU/tomato, respectively, on day 0 (Figure 4.1). Tomatoes yielding these counts were in contact with inoculated soil for only 10 min. There was ca. 2.5 $\log_{10}$ CFU/tomato increase of population during the first 4 days of storage. Counts remained constant between 4 and 10

FIGURE 4.1 *Populations of* Salmonella *on spot-inoculated tomatoes* (○, ●), *tomatoes placed on soil during storage* (□, ■), *and in soil on which tomatoes were placed during storage* (△, ▲). *Open and closed symbols indicate populations recovered on BSA and BHI/Amp agar, respectively. Values for populations in soil are presented as* log_{10} *CFU/g; values for populations on spot-inoculated tomatoes and tomatoes placed on inoculated soil during storage are presented as* log_{10} *CFU/tomato.*


9
8
7
6
5
4
3
2
1
0
Salmonella (log10 CFU)
0
2
4
6
8
10
12
14
Storage time (days)

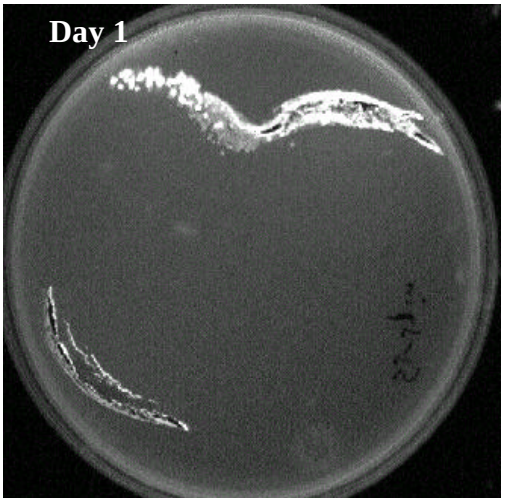
days. Tomatoes in contact with moist soil for 10 days were excessively decayed, and were not analyzed for population of *Salmonella* at 14 days.

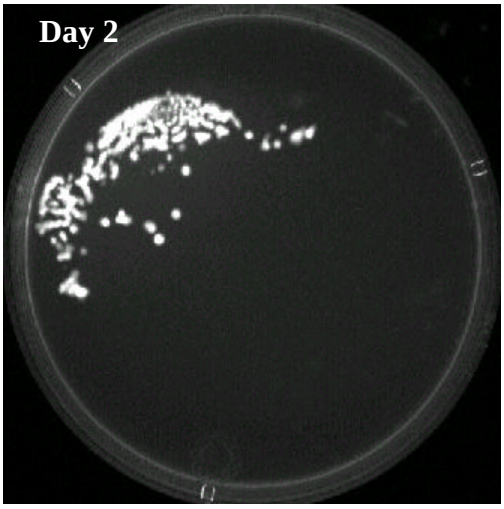
Survival of *Salmonella* in soil. The initial populations of *Salmonella* in inoculated soil recovered on BSA and BHI/Amp agar were 8.15 and 7.77 $\log_{10}$ CFU/g, respectively (Figure 4.1). Counts remained constant during the 14-day storage period. Statistical analysis showed that there was no significant difference between populations of *Salmonella* recovered on BSA and BHI/Amp agar from any of the soil samples. After 45 days, the population was 6.70 $\log_{10}$ CFU/g (data not shown).

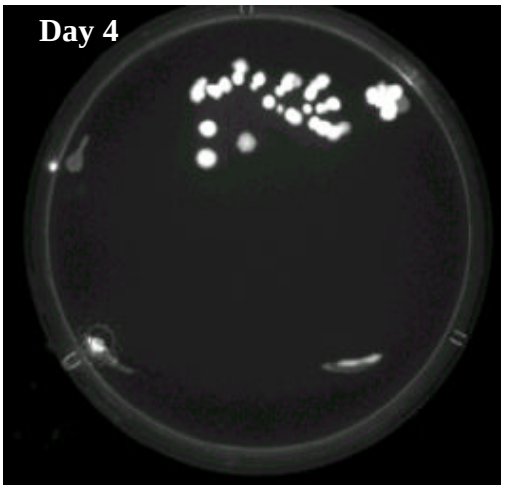
Distribution of *Salmonella* in cut tomatoes. Imaging of cut tomatoes revealed that, fluorescent cells appeared only near or on the surface of tomatoes stored on inoculated soil for 1 day (Figure 4.2). More fluorescence was observed in the stem scar and periphery area of pulp of cut tomatoes as the time of storage of tomatoes in contact with inoculated soil increased.

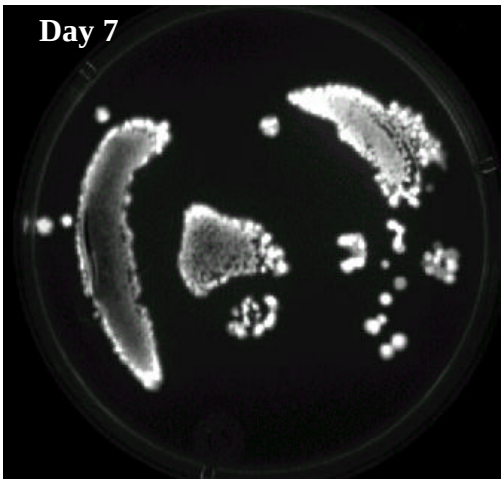
Confirmation of *Salmonella* isolates. Cells from selected presumptive *Salmonella* colonies were subjected to serotests and PCR fingerprinting. Serotesting revealed that PCR fingerprint profiles of isolates from tomatoes and soil matched those of stock cultures used to prepare inoculum. Representative PCR fingerprinting profiles of isolates from tomatoes and soils are shown in Figure 4.3. Among the *Salmonella* isolates from fifteen inoculated soil samples, *S.* Poona, *S.* Montevideo, *S.* Michigan, and *S.* Enteritidis were detected in 7, 4, 2 and 2 samples, respectively. *S.* Hartford was not detected. Of the isolates from fifteen tomatoes in contact with inoculated soil for 10 days, *S.* Montevideo, *S.* Enteritidis, *S.* Poona, and *S.* Michigan were detected in 8, 3, 2 and 2 samples, respectively. *S.* Montevideo was also the most dominant serotype (11 of 15 colonies) recovered from spot-inoculated tomatoes held for 14 days; *S.* Poona and *S.* Michigan were both detected in two samples; *S.* Hartford and *S.* Enteritidis were not detected.

FIGURE 4.2 *Distribution of* Salmonella *on and in tomatoes placed on the surface of soil inoculated with the pathogen. Whole tomatoes were cut in half and placed (cut side down) on BHI/Amp for 1 min, then discarded; plates were incubated 24 h before examining colonies for fluorescence. Photographs exemplify distribution of* Salmonella *near the surface or in subsurface tissue of tomatoes after storing on the surface of soil 1, 2, 4, 7, and 10 days.*


Day 1


Day 2


Day 4


Day 7

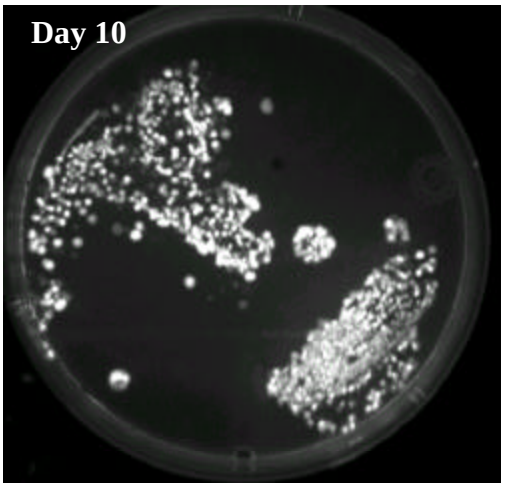
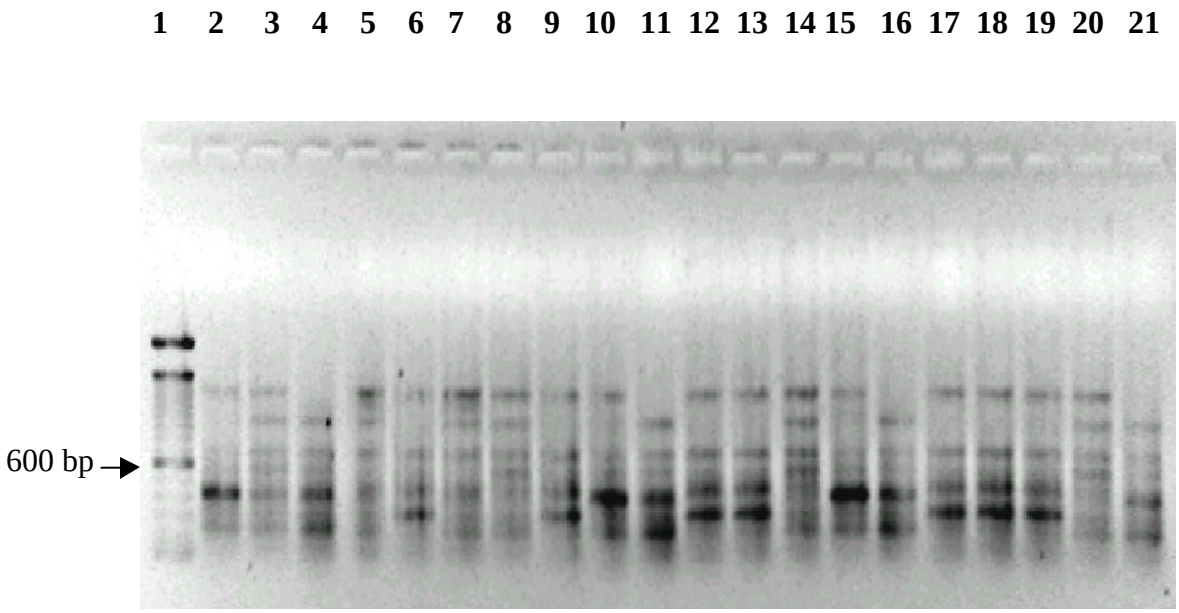

Day 10

FIGURE 4.3 *DNA-based typing of presumptive* Salmonella *colonies isolated from tomato fruits and soil. Lane 1, 100-bp DNA marker; lanes 2, 3, 4, 5, and 6, DNA profiles of S.* Enteriditis*, S.* Hartford, *S.* Michigan*, S.* Poona *and S.* Montevideo*, respectively; lanes 7 through 11, DNA profiles of isolates from inoculated soil; lanes 12 through 16, DNA profiles of isolates from tomato fruits placed on the surface of soil for 10 days; lanes 17 through 21, DNA profiles of isolates from tomato fruits spot inoculated with a water suspension of* Salmonella.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
600 bp

DISCUSSION

Results show that *Salmonella* survives at high numbers in moist soil for up to 45 days. Watkins and Sleath (35) demonstrated that *Salmonella* in sewage sludge applied to agricultural soils died off within 5 weeks, whereas *Listeria monocytogenes* survived for 8 weeks. Microorganisms deposited on flooded croplands may remain in soil for months or years (4).

The survival pattern of *Salmonella* serotypes on tomatoes, either spot-inoculated with a water suspension of the pathogen or contaminated by contact with inoculated soil, indicates that *S.* Montevideo is dominant and *S.* Hartford is absent after extended storage time. This is in agreement with a previous study (20) reporting that *S.* Montevideo was the most persistent serotype on ripe tomato fruits from plants inoculated at the time of flowering with the same mixture of *Salmonella* serotypes used in the present study. *S.* Montevideo was isolated from tomatoes 49 days after flowers were inoculated, and was the most dominant among serotypes in the inoculum.

Zhuang et al. (38) reported that the population of *S.* Montevideo on the surface of inoculated tomatoes stored at 10°C did not change significantly throughout an 18-day period; however, at 20°C, within 7 days, the population increased significantly, then decreased to 2 $\log_{10}$ CFU/cm^2 on day 18. *L. monocytogenes* was observed to grow on whole tomatoes held at 21°C but not at 10°C, although it remained viable for up to 20 days (3). In our study, the population of *Salmonella* on spot-inoculated tomatoes decreased by about 4 logs during the 14-day storage at 20°C. Differences in patterns of survival of *Salmonella* on tomatoes may be influenced by the method of inoculation. We used a spot inoculation procedure, which enables a known population of *Salmonella* to be applied, whereas Zhuang et al. (38) and Beuchat and Brackett (3) dipped tomatoes into a cell suspension. The latter procedure may result in cells becoming lodged in tissue areas imposing conditions that enhance survival and growth of cells during subsequent storage.

The distribution of *Salmonella* in cut tomato tissues suggested that, upon contact with moist inoculated soil, cells may infiltrate fruits through the stem scar and into the

pulp. Lin and Wei (22) observed that the distribution of *S.* Montevideo on the pulp surface of tomatoes cut with a knife was related to the inoculum dose applied to the stem scar. *S.* Montevideo colonies were clustered in the stem scar region of tomatoes receiving a low inoculum; colonies were more widely dispersed on cut pulp of tomatoes receiving a higher inoculum. Zhuang et al. (38) demonstrated that populations of *Salmonella* on dip-inoculated tomatoes are highest in the stem scar tissue. Other researchers have described infiltration of fruits with pathogenic bacteria. The greatest extent of infiltration of apples with *E. coli* O157:H7 was observed by Buchanan et al. (7) to be consistently associated with outer core region, followed by skin and pulp. Internalization of *E. coli* in apples subjected to natural environmental conditions has been studied by Seeman et al. (32). Apples were placed on artificially contaminated soil to mimic the fate of drop or windfall apples. The presence of *E. coli* in the inner core and flesh of apples indicated that internalization can occur under simulated orchard conditions. These studies and our observations on infiltration of *Salmonella* into tomatoes support the contention that preharvest contact of produce with contaminated water or soil exacerbates problems associated with postharvest removal or inactivation of pathogens.

Contamination of tomatoes with microorganisms can occur during fruit development and harvesting. Samish et al. (31) studied the distribution of bacteria within healthy tomato fruits. They observed that tomatoes from farms using overhead irrigation contained bacteria more frequently and at higher populations than tomatoes from farms using furrow irrigation. Overhead irrigation may produce a high-humidity microclimate more favorable for attachment or growth of some bacterial species on the surface of tomatoes. Armen and Ingham (1) studied the decline in populations of *E. coli* O157:H7 and *S.* Typhimurium DT104 in land-spread bovine manure exposed to various soils, temperatures, and moisture regimes. Their results demonstrated that bacterial death occurred more rapidly at 25^{o}C than at 10 ^{o}C; *E. coli* O157:H7 declined more rapidly than *S.* Typhimurium DT104 under similar inoculation conditions. Neither soil type nor

watering regime seemed to have appreciably affected the rate of decline. In contaminated agricultural environments, e.g. soil and water, *Salmonella* may attach to or enter tomato fruits and survive for an extended time. If there is no effective intervention step to kill or remove the pathogen, an increased risk of human infection would result.

We used EGFP as marker to study growth and survival of *Salmonella*. Although populations recovered on BSA were consistently higher that on BHI/Amp agar, there was no significant difference between counts from a given sample obtained on BSA and BHI/Amp agar after a given storage time. It was easier to differentiate the fluorescent colonies from background flora on BHI/Amp agar compared to BSA. However, lack of selective pressure may result in a loss of the EGFP plasmid, thus result in lower apparent numbers of *Salmonella* recovered on BHI/Amp agar. Prechaiyo and Mclandsborough (27) introduced EGFP into *E. coli* and showed that the expression of EGFP did not change growth kinetics or cell surface properties. However, they also noted a number of limitations using EGFP system, such as high levels of plasmid instability, which may be overcome by either including antibiotic selection or the construct *egfp* gene into the chromosome.

Our study demonstrates that soil and water are potential reservoirs of *Salmonella* for contaminating tomatoes. The pathogen can survive in moist soil at high populations for at least 45 days. Once attached to tomato fruits, *Salmonella* either survives or slowly dies. The pathogen may infiltrate tissues of tomatoes during contact with inoculated soil. Appropriate intervention steps to prevent contamination of tomatoes by soil and water containing *Salmonella* are critical if the risk of salmonellosis is to be minimized.

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CHAPTER V

UPTAKE OF *SALMONELLA* BY ROOTS OF TOMATO SEEDLINGS GROWN HYDROPONICALLY[1]

[1]Guo, X., M. van Iersel, J. Chen, R. E. Brackett, and L. R. Beuchat. To be submitted to *Journal of Food Protection*.

ABSTRACT

Outbreaks of infections associated with raw and minimally processed fruits and vegetables have occurred with increased frequency in the U.S. in recent years. The assumption has been that infectious microorganisms are on the surface of implicated produce. Several investigators, however, have reported isolating low numbers of non-pathogenic bacteria from internal tissues of apparently intact vegetables. In this study, we investigated the possibility of uptake of *Salmonella* by roots of tomato seedlings grown in Hoagland's nutrient solution, and transport of the pathogen to cotyledons, stems, and leaves. Within 1 day of exposing seedling roots to inoculated solution, populations of *Salmonella* were, respectively, 3.01 and 3.40 $\log_{10}$ CFU/g of the cotyledon and stem portions of plants with an intact root system (control), and 2.55 $\log_{10}$ CFU/g of cotyledons of seedlings from which a portion of the root was removed. *Salmonella* was detected at populations >3.38 $\log_{10}$ CFU/g of cotyledons, stems, and leaves of plants grown in inoculated nutrient solution for 9 days, regardless of the root condition. PCR analysis revealed that, among five serotypes in the inoculum, *S.* Montevideo predominant in all tissues of 9-day samples. Results indicate that in a controlled hydroponic system, *Salmonella* was transported from a nutrient solution to cotyledons, stems, and leaves through either intact or cut roots. Appropriate interventions need to be taken to prevent potential systemic contamination of tomato fruits.

INTRODUCTION

Outbreaks of infections associated with raw and minimally processed fruits and vegetables have occurred with increased frequency in the U.S. in recent years (NACMCF, 1999). Fresh produce, including tomatoes, seed sprouts, watermelons, and cantaloupes have been identified as potential vehicles of human salmonellosis (Mahon et al., 1997; O'Mahony et al., 1990; Van Beneden et al., 1999; Blostein, 1991; CDC, 1979, 1991, 1999; Gaylor et al., 1955; Ries et al., 1990; Wood et al., 1991).

Microorganisms are present largely on the surface of raw fruits and vegetables. The inner tissue of sound produce is generally considered to be sterile. However, numerous investigators have reported isolating low numbers of bacteria from internal tissues of apparently intact vegetables (Lund, 1992; Robbs et al., 1996). Samish et al. (1962) studied ten fruits and vegetables grown on different farms and found that bacteria, mostly species of gram-negative motile rods in two families, Pseudomonadaceae and Enterobacteriaceae, occurred within normal, sound raw cucumber and tomato fruits.

Hydroponically grown radish sprouts have been epidemiologically implicated as a vehicle for transmitting *Escherichia coli* O157:H7 infection (WHO, 1996). Hara-Kudo et al. (1997) studied distribution of the pathogen on radish sprouts grown in inoculated water. The cotyledons and hypocotyl were externally contaminated with the pathogen when sprouts were grown from seeds soaked in water containing *E. coli* O157:H7 at a population of 3.0 to 3.1 $\log_{10}$ CFU/ml. The pathogen was detected in cotyledons and hypocotyls within 18 h of exposure of roots to inoculated water. Itoh et al. (1998) detected viable *E. coli* O157:H7 in inner tissues and stomata of cotyledons of radish sprouts grown in an aqueous suspension of the pathogen. Gandhi et al. (2000) used *E. coli* and *Salmonella* Stanley tagged with green fluorescent protein (GFP) to investigate spatial location within alfalfa sprouts. Microscopy revealed the presence of marker bacteria in subsurface areas of root, hypocotyl, and cotyledon tissues.

It is essential that interventions be developed to prevent or minimize contamination of raw produce and to kill or remove human pathogens prior to consumption in order to

minimize risk of human infections. To date, however, none of the chemical or physical treatments currently authorized by regulatory agencies for use to disinfect raw produce can be relied on to eliminate all types of pathogens from the surface or internal tissues (Beuchat, 1998). Devising successful intervention steps to reduce populations of human pathogens on and in fruits and vegetables will be aided by knowledge of the sources of contamination and the ecology of pathogens as affected by agronomic and processing practices (Beuchat, 1998; Brackett, 1999; Buchanan et al., 1999).

Bacteria with a fluorescence marker such as GFP can be used to facilitate monitoring survival, growth, and colonization under various ecological conditions. Most of the plant research using GFP-tagged bacteria has involved intact plant tissues. Fluorescent protein also has been used to monitor and study infectious plant pathogens (Leffel et al., 1997), as a replacement for protein coating on potato virus X (Baulcombe et al., 1995), and to study movement of the virus through and among host plants (Oparka et al., 1996).

We hypothesized that human pathogens such as *Salmonella* may attach to or infiltrate tomato plant tissues at various preharvest stages of fruit development. In a previous study (Guo et al., 2001a), we observed that *Salmonella* inoculated onto tomato flowers and into stems survived for at least 49 days and was present on and in ripened fruits. The objectives of the study reported here were to investigate the possibility of uptake of *Salmonella* by intact and cut roots of tomato seedlings, and transport to cotyledons, stems, and leaves of seedlings grown in a nutrient solution inoculated with the pathogen.

MATERIALS AND METHODS

Bacterial cultures. Five serotypes of *Salmonella* were used: *S*. Montevideo (serogroup C_1) was isolated from a patient in a tomato-associated outbreak, *S.* Michigan (serogroup J) was isolated from cantaloupe, *S*. Poona (serogroup G) was isolated from a patient in a cantaloupe-associated outbreak, *S*. Hartford (serogroup C_1) was isolated from a patient in an orange juice-associated outbreak, and *S*. Enteritidis (serogroup D) was

from a patient in an egg-associated outbreak. Transformation of each serotype with plasmid pEGFP (enhanced green fluorescent protein) (Clonetech Laboratories, Inc., Palo Alto, Calif.) was done according to procedures described by Guo et al. (2001b).

Inoculum preparation. Five serotypes of *Salmonella* labeled with pEGFP were used to prepare inoculum as described previously (Guo et al., 2001). Inoculum (4 ml) was added to 4 liters of half-strength modified Hoagland's nutrient solution (Hoagland and Arnon, 1950) supplemented with ampicillin (100 μg/ml) (Sigma, St. Louis, Mo.) to give a population of 4.55 ± 0.10 $\log_{10}$ CFU/ml.

Tomato seedlings. 'Better boy' cv. tomato seeds were purchased from Park Seed Company, Greenwood, S.C. Sand (QUIKRETE® Companies, Atlanta, Ga.) was used as a medium to germinate seeds and grow plants to a height of 4 cm. Sand was moistened with water and placed in a 3-cm layer in a polycarbonate tray (50 cm long × 30 cm wide × 15 cm deep) (Nalge Company, Rochester, N.Y.). Tomato seeds were placed on the surface, covered with a 0.5-cm layer of sand, and held in the dark at 25°C in a forced-air incubator for 7 days, or until cotyledons emerged.

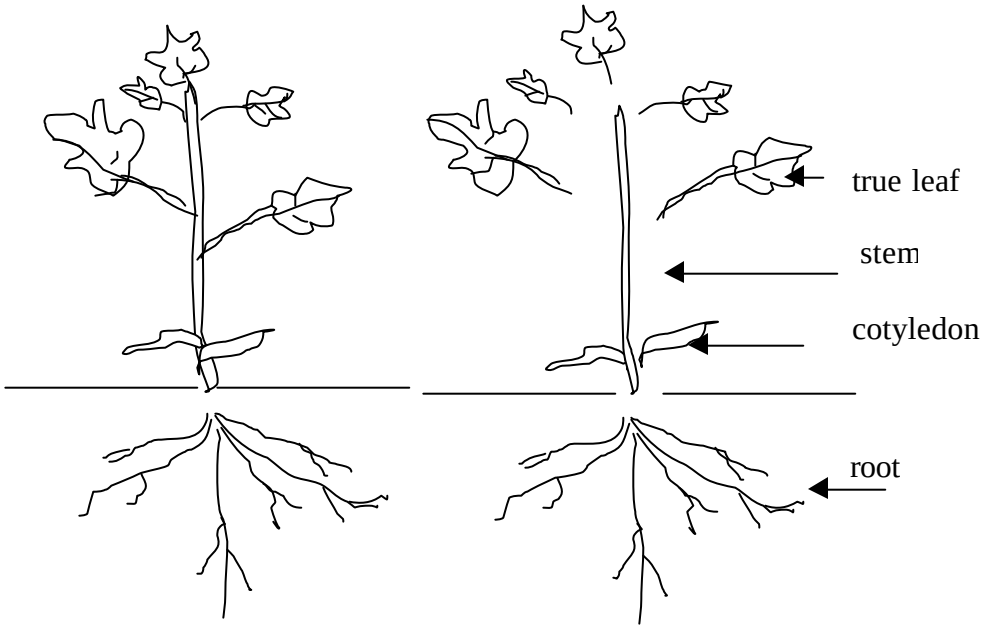
Hydroponic system. The hydroponic system was set up in a walk-in incubator at 25°C. Eight polycarbonate hydroponic trays (27 cm long × 21 cm wide × 14 cm deep), each designed to hold 12 seedlings, were used. The sides were covered with aluminum foil to shield the Hoagland solution from light. Hoagland's nutrient solution was prepared using ingredients purchased from Fisher Scientific (Pittsburgh, Pa.). A sheet of styrofoam (2.5 cm thick) containing holes (1 cm diameter) was placed 1.5 cm above the surface of half-strength Hoagland's solution (4 liters, 12 cm deep) in hydroponic trays. Seedlings (4 cm high) from which sand was removed from the roots by washing with water were placed in holes and secured with nonadsorbant cotton such that the root was below the styrofoam and the cotyledons, stem, and leaves were above. Hoagland's solution was aerated using air stones connected to an aquarium air pump (Tetra/Secondnature, Blacksburg, Va.) using tubing and splitters. Sets of four trays were

enclosed within perforated plastic film to minimize air flow. Wide spectrum fluorescent bulbs (ca. 80 μmol/m^2/s) for plants and aquaria (General Electric Company, Cleveland, Ohio) were used as a light source for tomato plants. A 16 h/8 h light/dark cycle was used. The pH of Hoagland's solution was monitored daily and maintained at 5.5 - 6.5 by replenishing with new solution.

Uptake of *Salmonella* by roots. After growing seedlings 7 days in Hoagland's solution, the bottom portion (1 cm) of the root system (5 cm long) of half of the seedlings was removed by cutting with a sterile scalpel. Plants with intact or cut roots were then transferred to trays containing 4 liters of Hoagland's solution inoculated with a five-serotype mixture of GFP-tagged *Salmonella*. Seedlings were positioned in holes in a sheet of styrofoam 1.5 cm above the inoculated Hoagland's solution. After three true leaves emerged, the cotyledons, stems, and leaves above the styrofoam sheet were severed from the roots (Figure 5.1). The severed portions from each plant were analyzed for the presence of *Salmonella*.

Microbiological analysis. Samples (0.11 - 2.21 g) of seedling cotyledons, stems, leaves of each plant were placed individually in sterile plastic bags (16 cm × 10 cm) containing 5 ml of sterile 0.1% peptone water, macerated by hand, and mixed for 1 min. Three plants were analyzed for each set of experimental parameters. Samples of macerates were surface plated (0.25 ml in quadruplicate) on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) supplemented with ampicillin (100 μg/ml) (BHI/Amp agar). Plates were incubated at 37°C for 24 h before examining for presumptive colonies of *Salmonella*. The remaining macerate was enriched by adding 5 ml of universal preenrichment broth (Difco) and incubating the mixture at 37°C for 24 h. Cultures were streaked on BHI/Amp agar and incubated at 37°C for 24 h before examining for presumptive *Salmonella* colonies using a UV light. Five fluorescent colonies from each sample were randomly picked and subjected to serological testing and ERIC PCR analysis. Serological identification was performed using *Salmonella*

FIGURE 5.1 *Schematic drawings of tomato seedling showing an intact plant (left) and portions of a plant (leaves, stem, cotyledons) analyzed for the presence and populations of* Salmonella. *Leaves, stem, and cotyledons from a single plant represented three separate samples.*


true leaf
stem
cotyledon
root

antiserum for groups C_1, D_1, G, and J (Difco) according to the manufacturer's instructions.

Confirmation of presumptive *Salmonella* colonies using PCR. PCR fingerprinting was done to compare serotypes of isolates from tissues collected 9 days after initial exposure to *Salmonella* to those in the inoculum. The primer used for PCR fingerprinting was (5'-3') AAG TAA GTG ACT GGG GTG AGC G, based on a highly conserved, enterobacterial repetitive intergenic consensus (ERIC) sequence which consists of 126 base pairs and appears to be restricted to transcribed regions of the genome, either in intergenic regions of polycistronic operons or in untranslated regions upstream or down stream of open reading frames (Hulton et al., 1991). Crude DNA was prepared by boiling 20-h cultures of isolates in BHI broth for 10 min. One milliliter of a 20-h culture grown in BHI broth at 37°C was centrifuged at 12,000 × *g* for 2 min. Pellets were resuspended in 200 µl of sterile distilled water, boiled for 10 min, and centrifuged at 12,000 × *g* for 2 min. A 5-µl sample was used as a template for PCR. The 50-µl PCR reaction mixture contained PCR buffer, dNTP (0.4 mM each), primers (1µM), Taq polymerase (1U, Roche Diagnostics, Indianapolis, Ind.), and DNA template. PCR reactions were performed in a DNA thermal cycler 480 (Perkin Elmer, Norwalk, Conn.) using a cycle at 94°C for 5 min, followed by 40 cycles of 92°C for 45 sec, 25°C for 1 min, and 68°C for 10 min, with a final extension at 72°C for 20 min. The PCR amplicons were analyzed by gel electrophoresis on 1% agarose (GIBCO BRL, Rockville, Md.) gel in TBE buffer (0.089 M Tris-borate, 0.002 M EDTA, pH 8.0). The gel was stained with ethidium bromide and visualized using a Gel Doc System 2000 (Bio-Rad Laboratories, Hercules, Calif.).

RESULTS

The population of *Salmonella* in inoculated Hoagland's solution was 4.46 – 4.65 $\log_{10}$ CFU/ml throughout the 9-day period that tomato seedlings with intact and cut roots were grown and analyzed for the presence of the pathogen in cotyledons, stems, and

leaves. Within 1 day of exposing seedling roots to inoculated solution, populations of *Salmonella* were 3.01 and 3.40 $\log_{10}$ CFU/g, respectively, in the cotyledon and stem portions of plants with an intact root system (control), and 2.55 $\log_{10}$ CFU/g of cotyledons of seedlings from which a portion of the root was removed (Table 5.1); *Salmonella* was also detected in enriched stems of plants with part of the root removed. The pathogen was not detected in leaves of seedlings exposed to inoculum for 1 day, regardless of root treatment. On days 3 through 9, with the exception of the stem portion on day 3, *Salmonella* was detected in the cotyledon and stem portions of control seedlings. *Salmonella* was detected in enriched leaves of control plants on day 3, but not in leaves of plants with a portion of the root removed until day 5. In stems of seedlings with severed roots, *Salmonella* was detected by enrichment on day 1 but not on days 3 or 5, and at a population of 1.14 $\log_{10}$ CFU/g on day 7.

Analysis of seedling tissues by PCR (Figure 5.2) revealed that, among the five serotypes inoculated into Hoagland's solution, *S.* Montevideo (8 of 10 isolates) and *S.* Michigan (2 of 10 isolates) were dominant. *S.* Montevideo and *S.* Michigan were identified, respectively, as 9 of 10 and 1 of 10 isolates from cotyledons analyzed on day 9 of the experiment. *S.* Montevideo was also detected in stems and leaves on days 9. *S.* Enteritidis, *S.* Hartford and *S.* Poona were not detected in tissues of any of the 9-day samples.

DISCUSSION

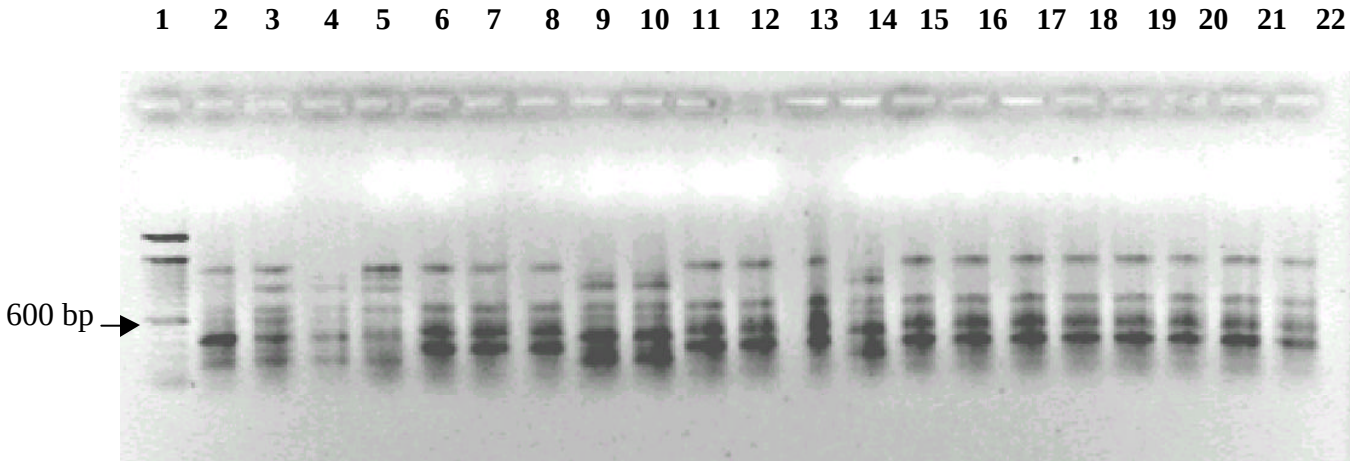
Salmonella survived in Hoagland's nutrient solution supplemented with 100 µg/ml ampicillin, which helped to maintain selective pressure and, thus, the stability of expression of EGFP. Within 24 h, uptake of *Salmonella* by intact and cut roots was evident from the presence of the pathogen in cotyledons. This agrees with a study on radish sprouts grown in a cell suspension of *E. coli* O157:H7 in which the pathogen was detected in cotyledons and hypocotyls within 18 h (Hara-Kudo et al., 1997). As the 9-day growing period progressed, although not consistent from one sampling time to the

TABLE 5.1 *Populations and presence of* Salmonella *in tissue samples of hydroponically grown tomato seedlings*

Root treatment	Tissue Analyzed	Time (days) after exposure of seedling roots to *Salmonella* in Hoagland solution											
		0		1		3		5		7		9	
		log_{10} CFU/g	E[a]	log_{10} CFU/g	E	Log_{10} CFU/g	E	log_{10} CFU/g	E	log_{10} CFU/g	E	log_{10} CFU/g	E
Intact	Cotyledons	-	-	3.01	+	2.70	+	2.48	+	>4.37	+	>4.02	+
	Stem	-	-	3.40	+	<1.53	-	2.01	+	2.06	+	>3.70	+
	Leaves	-	-	<1.22	-	<1.28	+	<0.98	+	1.06	+	>3.61	+
Cut	Cotyledons	-	-	2.55	+	1.89	+	1.44	+	>4.12	+	>4.29	+
	Stem	-	-	<1.32	+	<1.30	-	<1.12	-	1.14	+	>3.58	+
	Leaves	-	-	<0.97	-	<0.97	-	1.08	+	<0.47	-	>3.38	+

[a]Enriched sample: + (present), - (absent)

FIGURE 5.2. *DNA-based typing of presumptive* Salmonella *colonies isolated from tomato seedlings grown in Hoagland's solution inoculated with a five-serotype mixture of the pathogen. Lane 1, 100-bp DNA marker; lanes 2, 3, 4, 5, and 6, DNA profiles of S.* Enteriditis, *S.* Hartford, *S.* Michigan, *S.* Poona, *and S.* Montevideo, *respectively; lanes 7 through 10, profiles of isolates from inoculated Hoagland solution; lanes 11 through 14, profiles of isolates from the cotyledons of tomato seedlings on day 9; lanes 15 through 18, profiles of isolates from stems of tomato seedlings on day 9; lanes 19 through 22, profiles of isolates from leaves of tomato seedlings on day 9.*


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
600 bp

next and in low populations, *Salmonella* was detected in stems and leaves of seedlings with intact or cut roots. Our previous study showing that *Salmonella* survives on and in tomato fruits during development supports the notion of systemic transport of the pathogen.

PCR fingerprinting revealed the same trend in survival of serotypes observed in a previous study, in which the prevalence of the same serotypes on and in tomato fruits produced on plants inoculated at the time of flowering was observed (Guo et al., 2001a). In both studies, *S.* Montevideo was the most dominant serotype isolated from tomato tissues.

An advantage of hydroponic produce, compared to conventional field-grown produce, is that plants are not in contact with soil that can serve as a source of pathogenic contaminants. Problems of disease and pests as well as soil, salinity, poor structure, and drainage are also minimized using hydroponic systems. In this study, the hydroponic system provided a controlled environment to study the uptake of *Salmonella* by roots, with minimal concern about environmental contamination or temperature fluctuation. Results indicate that, in a controlled hydroponic system, *Salmonella* was transported from a nutrient solution to cotyledons, stems, and leaves through either intact or cut roots. Appropriate steps need to be taken to prevent potential systemic contamination of tomato fruits.

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CHAPTER VI

SUMMARY AND CONCLUSIONS

Outbreaks of infections associated with raw and minimally processed fruits and vegetables have occurred with increased frequency in the U.S. in recent years. The assumption has been that infectious microorganisms are on the surface of implicated produce. Several investigators, however, have reported isolating low numbers of bacteria from internal tissues of apparently intact vegetables. We hypothesized that human pathogens such as *Salmonella* may migrate into plant tissues at some point during preharvest production and postharvest handling. Tomato was used as a model to investigate conditions that influence migration, growth, survival, and death of *Salmonella* in plant tissues.

A *hilA*-based PCR assay was developed and evaluated on eighty-three *Salmonella* and twenty-two non-*Salmonella* strains, and validated for detecting *Salmonella* Montevideo in and on inoculated tomatoes, and can be used for rapid detection of *Salmonella* in or on tomato fruits.

The establishment of *Salmonella* in tomato plants through different routes before harvest, such as uptake of *Salmonella* by roots of tomato seedlings grown in Hoagland's nutrient solution, stem injection, and flower brushing during fruit development in a greenhouse, was investigated. In a controlled hydroponic system, *Salmonella* was transported from a nutrient solution to cotyledons, stems, and leaves through either intact or cut roots. The pathogen survives in or on tomato fruits from the time of inoculation at flowering through fruit ripening. Tomato stems and flowers are possible sites at which *Salmonella* may establish and remain viable during fruit development, thus serving as routes or reservoirs for contaminating ripened fruit.

A postharvest storage study demonstrated that soil and water are potential reservoirs of *Salmonella* for contaminating tomatoes. Once on the surface of tomato fruits, *Salmonella* either survives or slowly dies. Observations on infiltration of *Salmonella* into tomatoes support the contention that preharvest contact of produce with contaminated water or soil exacerbates problems associated with postharvest removal or inactivation of the pathogen.

Our study reveals its ability to survive on and in tomato fruits throughout the course of plant growth, flowering, fruit development and maturation, and postharvest storage. Tomato stems and fruits are subject to mechanical injury in the field and during postharvest handling, which would likely make them more susceptible to internalization of the pathogen. Thus, appropriate steps need to be taken to prevent potential systemic contamination of tomato fruits via irrigation water, manure, soil, water used to apply fungicides and insecticides, and handling in an attempt to prevent or minimize access of *Salmonella* to the tomato plants and fruits at various points from the farm to the consumer.