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Prevalence of *Salmonella*, *Escherichia coli* and coliforms on bell peppers from the field to the packing house process

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The presence of *Salmonella* spp., *Escherichia coli* and coliforms from the field to the packing process of bell pepper was evaluated. A total of 900 samples including bell pepper, worker hands, surfaces, and hand-washing fruits were collected from 11-sampling sites of a farm. From the total samples analyzed, 2.6, 3.0, and 53.7% contained *Salmonella* spp., *E. coli* and coliforms, respectively. The highest percentage of positive samples with *Salmonella* and *E. coli* occurred at the field and packing house, respectively; while coliforms levels increased, as it approached to the final packing process. *E. coli* O157:H7 was not detected in any of the analyzed samples, however *Salmonella* enterica serovar Typhimurium was found during the packing process of the produce. Good agricultural practices from the field to the packinghouse should be implemented; it is also imperative to emphasize hand washing and contact surface disinfection to ensure the safety of the final produce.

Key words: Microbial tracking source, *Salmonella*, hand washing, surface contact, packinghouse, bell pepper (*Capsicum annuum*).

INTRODUCTION

Fresh produce production, particularly from Latin American countries, has the potential to meet most of the growing global demand for fruit and vegetable products. There is a need to increase food production to feed an

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ever-growing world population, as FAO has predicted that global food production will need to increase by 50% over current levels by 2050 (FAO, 2009). However, the globalization of the food supply may introduce new food safety risks and the potential widespread dissemination of contaminated food. Even thought, produce-related outbreaks in the United States declined by 42% over a 10–year period from 2002-2011, a sharp increase in produce-related foodborne illnesses occurred in 2008, due to a large multi-state *Salmonella* outbreak involving peppers and tomatoes that sickened over 1,535 people (CSPI, 2014). *Salmonella* enterica serovar Saintpaul was the causative agent of this outbreak, and was isolated from serrano and jalapeño peppers from two packinghouses in Tamaulipas, Mexico (Mody et al., 2011). Since then, the FDA has documented different commodities in Mexico contaminated with *Salmonella* spp. including cucumber, jalapeño peppers, serrano peppers, papaya, spinach, Mangoes and coriander (CDC, 2012; FDA, 2012). Additionally, two more documented outbreaks in which cantaloupes and peppers were implicated proved that *Salmonella* could have originated from field and farm operations in Mexico (CDC, 2002; CDC, 2008). Several others tracking-type investigations have located *Salmonella* and *E. coli* O157:H7 in different points of the food production chain such as water, contact surfaces, fresh produce (melon, orange, parsley and bell pepper), worker hands, animals, and soil (Castillo et al., 2004; Mukherjee et al., 2004; Duffy et al., 2005; Gallegos-Robles et al., 2008; Cooley et al., 2014).

As the overall consumption of fresh produce has increased, the amount of produce imported into the U.S. market has also increased. In 2008, nearly 3 million metric tons of fresh vegetables were imported from Mexico to the USA, representing more than one-half of all revenues from US vegetable imports (ERS, 2009; FAS, 2009). Culiacan is the capital of Sinaloa and is located in the Northwestern part of Mexico. This particular region is rich in vegetable produce farms that export several crops to the United States each year, particularly tomatoes and bell peppers (CIAD, 2006; Estrada-Acosta et al., 2014). In an effort to examine microbial contamination in this region, a comprehensive study to determine the presence of bacterial pathogens and the identification of critical points during the production chain is needed. Therefore, to address these data needs, the goals of this study were: 1) to evaluate the incidence of *E. coli*, coliforms, and *Salmonella* spp. from the harvesting to the final packaging process of bell pepper, and 2) to characterize the isolated strains of *E. coli* and *Salmonella* spp.

MATERIALS AND METHODS

Sampling collection

Samples were weekly collected during the growing season of November 2006 to December 2007 from an agricultural packing-house operation in the Northwestern region of Mexico. The packinghouse owner in the study gave its consent to participate and to collect samples during the growing season. Samples were collected from the field and the packinghouse facility including fresh fruits, food contact surfaces, hand washing water, and worker hands. Table 1 shows the number of samples collected and locations from each commodity. Fresh produces were collected directly from the plant during harvesting, and from the field containers at the field. In the packinghouse, produce samples were collected from the unloading ramp, brushing, sorting, and waxing. Finally, produces were also collected from the packing bins and packing containers. Samples from worker hands (picker, sorter and packer) were collected both before and after 3h of continue labor. The contact surface areas of the packinghouse equipment were taken from the unloading ramp, roller, conveyor belts, and packing bin. All samples were stored at 5°C and transported to the Centro de Investigacion en Alimentacion y Desarrollo Laboratory in Sinaloa, Mexico for immediate processing. Microbial analyses were initiated within 24h of sample collection.

Microbiological sampling procedure

Each sample was collected and analyzed as follows: fresh produces from each location were obtained using sterile, disposable gloves and were individually placed into Ziploc® bags previously sterilized with UV light. 195 mL of buffer peptone water 2% (BPW) (Difco) were added to each 100 g sample of bell pepper. Each worker rinsed their hands during 30 s in 200 mL of sterile phosphate buffered saline (PBS) at pH 7. Then, 10 mL of BPW (Difco), were added to 40 mL of PBS and stored at 5°C until analysis. Approximately, 2,500 cm^2 of each contact surface was swabbed using a sterile sponge (Whirl Pack® Hydrated Speci-Sponge® Bag) of 3.8 x 7.6 cm in 5 mL of BPW (Difco), followed by addition of 195 mL of BPW 2%. Finally, 800 mL of hand washing water were collected in a 1 L sterile plastic bottle, and 2mL of thiosulphate at 10% were added to neutralize any chlorine presence. All samples were manually homogenized and processed for enumeration of *E. coli*, coliforms and *Salmonella* spp. as described by APHA (2001).

Escherichia coli and coliforms

Quantification of *E. coli* and coliforms was performed using the membrane filtration technique (APHA, 2001). Aliquots of 1 and 10 mL of BPW from each bell pepper produce and sponge samples were added to 50 mL of sterile distilled water and filtered by a cellulose membrane of 47 mm in diameter and 0.45 µm pore size (GN-6 Metricel®, Pall Corp., NY, USA). For each hand washing water sample 100 mL were filtered under the same conditions. After filtration, each membrane was placed on ECC agar (CHROMagar™ECC, Paris, France) and the plates were incubated at 42.5°C for 24 h. Colonies were quantified according to its morphological characteristics: *E. coli* and Coliforms-like colonies (blue and mauve colonies, respectively). A recovery efficiency of ≤1 CFU/mL was calculated for this experiment. Additionally, from the medium selective plate, two or more *E. coli* colonies were transferred onto tryptic soy agar (TSA) (Difco) and incubated at 35°C for 24 h. The isolates were stored at -20°C in glycerol (10%) for further molecular analysis.

Salmonella spp. enrichment and isolation

The same BPW culture used for *E. coli* isolation was used for *Salmonella* culturing according to Castillo et al. (2004) with some modifications. Briefly, 70 mL of the bell pepper produce pre-enrich-

Table 1. Number of samples classified by locations.

Sample type	Source	Number	Sample location
Bell peppers (N=568)	Field	66	Plant
		63	Harvest
		61	Field containers
	Packinghouse	63	Unloading ramp
		62	Brushing
		61	Sorting
		60	Waxing
		66	Packing bin
		66	Packing container
Workers hands (N=186)	After washing hands (N=98)	36	Picker
		31	Sorter
		31	Packer
	After 3 h of labor (N=88)	38	Picker
		27	Sorter
		23	Packer
Surface contact area (N=132)	Packinghouse equipment	33	Unloading ramp
		33	Roller
		33	Conveyor belts
		33	Packing bin
Hand washing water (N=14)	Packinghouse	14	Water
Total		900	

ment broth was incubated at 37°C for 24 h. For each surface contact and hands sample, 10 mL BPW was added to 90 mL of universal pre-enrichment broth (UPB) (Difco) and incubated at 37°C for 24 h. For each water sample, a volume of 100 mL was filtered through a cellulose membrane of 47 mm diameter and 0.45 µm pore size (GN-6 Metricel®, Pall Corp., NY, EUA), and placed in 8 mL of UPB, stirred for 1 min and incubated at 37°C for 24 h. For enrichment, 1 mL of the pre-enriched BPW or UPB were added to 10 mL tetrathionate broth (TTB) (Difco) and incubated at 42.5°C for 6 h followed by post-enrichment adding 1 mL of enriched TTB to 10 mL of M broth (Difco) and incubated at 37°C for 24 h. The post-enriched M broth-PCR positive samples were streaked on xylosa desoxycholate (XLD) Agar (Bioxon) and incubated at 37°C for 24 h. The method showed a recovery efficiency of ≤1 CFU/g and ≤1 CFU/mL.

Salmonella spp. detection and confirmation

Two or more *Salmonella* spp. presumptive colonies were isolated and identified by molecular analysis (a total of 50 isolates). PCR was performed according to Chiu and Ou (1996) with some modifications noted below. The DNA template was produced as follows: 1.5 mL post-enrichment M broth was centrifuged (13,200 x *g* for 5 min). The pellet was suspended twice in 400 µL sterile nanopure water and centrifuged under the same conditions listed above. The pellet was suspended in 200 µL of sterile nanopure water and heated for 5 min in boiling water to lyse bacterial cells and release DNA. The presence of *Salmonella* spp. was evaluated in lysed cells using the PCR, described as follows, the primers used were INVA-1 (5'-ACAGTCCTCGTTTACGACCTGATT-3´) and INVA-2 (5'-AGACGACTGGTACTGATCGATATT-3') that corresponds to a specific region of virulence *inv*A gene of *Salmonella* spp. of 244 bp. The PCR mixture (25 µL) consisted of 1X PCR amplification buffer (Promega, Madison WI), 1.5 mM $MgCl_2$, 400 µM dNTPs, primers INVA-1 and INVA-2 1 µM each, 1.25 U *Taq* polymerase (Promega), 13.875 µL of nanopure water and 1 µL of lysed cells. The PCR amplification conditions was produce as follows: one cycle at 94°C for 10 min, 30 cycles consisting of 95°C for 30 s to denature DNA, 56°C for 0.5 min to align the DNA primers and 72°C for 2 min for DNA extension. Amplification was performed in an Eppendorf™ thermocycler. PCR products were visualized by electrophoresis with tris-acetate-EDTA buffer in 1% agarose gel stained with ethidium bromide. Finally, the PCR-positive isolates for *Salmonella* spp. were sent to the Bacteriology Department at National Institute of Epidemiological Diagnosis and Reference in Mexico City for serotyping.

Detection of pathogenic *Escherichia coli*

Isolates were examined and screened by PCR according to López-Saucedo et al. (2003) as follows: DNA template was extracted from suspended cells grown on TSA, cleaned in nanopure water and lysed at 100°C. The primers used to identify pathogenic strains of *E. coli* were *lt* and *st* for enterotoxigenic *E. coli* (ETEC), *bfp*A and *eae*A for enteropathogenic *E. coli* (EPEC), *eae*A and *stx*1 for enterohemorrhagic *E. coli* (EHEC) and, *stx*2 and *ial* for enteroinvasive *E. coli* (EIEC), which originated DNA fragments of 450, 190, 324, 384, 150, 255 and 650 pb, respectively (López-Saucedo et al*.,* 2003). The PCR mixture consisted of 1X buffer, $MgCl_2$ 1.85 mM, dNTPs 184 µM, primers *lt* (5.0 pM), *st* (6.47 pM), *bfp*A (2.5 pM), *eae*A (3.88 pM), *stx*1 (3.88 pM), *stx*2 (2.5 pM), *ial* (10.25 pM), 0.625 U of DNA *Taq* polymerase (Promega), 2 µL of

Table 2. Presence of *Salmonella* spp., *Escherichia coli* and coliforms on samples collected in a bell pepper packinghouse.

Source	No. of positive samples (%)†		
	Salmonella spp.††	*Escherichia coli*	Coliforms
Bell peppers	15 (2.6%)	17 (3.0%)	305 (53.7%)
Worker hands	25 (13.4%)	24 (12.9%)	121 (65.1%)
Surface contact area	5 (3.8%)	9 (6.8%)	92 (69.7%)
Hand washing water	0 (0.0%)	2 (14.3%)	5 (35.7%)
Total (N=900)	45(5.0%)	52 (5.8%)	523 (58.1 %)

†Percentage of positive samples based on total samples analyzed; ††Positive samples by PCR.

Table 3. Presence of *Salmonella* spp., *Escherichia coli* and coliforms on bell peppers collected from the field and packinghouse.

Source of bell pepper sample	No. of samples analyzed	No. of positive samples (%)†		
		Salmonella spp.††	*Escherichia coli*	Coliforms
Plant	66	0 (0.0%)	0 (0.0%)	14 (21.2%)
Harvest	63	6 (9.5%)	1 (1.6%)	18 (28.6%)
Field containers	61	5 (8.2%)	1 (1.6%)	26 (42.6%)
Unloading ramp	63	2 (3.2%)	3 (4.8%)	38 (60.3%)
Brushing	62	0 (0.0%)	3 (4.8%)	39 (62.9%)
Sorting	61	1 (1.6%)	3 (4.9%)	38 (62.3%)
Waxing	60	0 (0.0%)	4 (6.7%)	41 (68.3%)
Packing bin	66	1 (1.5%)	2 (3.0%)	48 (72.7%)
Packing container	66	0 (0.0%)	0 (0.0%)	43 (65.2%)
Total	568	15 (2.6%)	17 (3.0%)	305 (53.7%)

†Percentage of positive samples based on total samples analyzed; †† Positive samples by PCR.

lysed cell and nanopure water to reach a total volume of 25 µL. The amplification was performed in an Eppendorf™ thermocycler with the following cycling conditions: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 5 min, 45 cycles to denature DNA at 95°C for 45 s, primers aligning at 50°C for 45 s and DNA extension at 72°C for 45 s; and a final extension step at 72°C for 10 min. The PCR products were visualized by electrophoresis through 2.5% agarose gel stained with ethidium bromide (López-Saucedo et al., 2003).

Statistical analysis

Descriptive statistics were performed to quantify the presence of *E. coli*, coliforms, and S*almonella* spp. using Minitab version 14 (MINITAB version 14.1, 2003).

RESULTS AND DISCUSSION

A total of 900 samples were collected from the field to the packinghouse during the growing season of November 2006 to December 2007. Among the 900 samples, 568 were bell pepper produces, 186 worker hands, 132 surface contact areas and 14 hand washing water. Table 1 specifies the number of samples classified by location. From the total samples analyzed. 5.0, 5.8, and 58.1% showed contamination with *Salmonella* spp., non-pathogenic *E. coli* and coliforms, respectively.

E. coli and coliforms were found in 14.3 and 35.7%, of the hand washing water samples (Table 2), with concentrations ranging from 7.1±1.6 to 17.2±41.2 log CFU/100mL, respectively. The study showed higher levels of coliforms than the one specified by the Mexican Official Norm (NOM-127-SSA-1994), which establishes 0 CFU/100mL. Thus, making the water neither acceptable for human consumption nor for hand washing due to possible cross contamination. On the other hand, *Salmonella* was not detected in any of the hand washing water samples analyzed.

In order to identify critical points of contamination on fresh produces, microbial levels on specific sampling locations from the field to the packinghouse were analyzed (Table 1). Coliforms were detected in 53.7% (Table 2) of the fresh produce sample locations with means ranging from 1.29 to 2.24 log CFU/100g. It was shown that coliform levels on fresh produce increased from the field and throughout packing, with 21.2% positive samples in the fresh produces obtained before detached from the plant, 42.6% in the container from harvest to field, and 60.3% from the field containers to the unloading ramp (Table 3). Coliforms levels showed an increased in the brushing and waxing step (62.9 and

Table 4. Presence of *Salmonella* spp., *Escherichia coli* and coliforms on worker hands from the field and packinghouse.

Source of worker' hands sample		No. of positive samples (%)[†]		
Sampling time	**Operator**	***Salmonella* spp.[††]**	***Escherichia coli***	**Coliforms**
Washing hands before labor	Picker	5 (13.9%)	0 (0.0%)	16 (44.4%)
	Sorter	6 (19.3%)	10 (32.3%)	27 (87.1%)
	Packer	6 (19.3%)	5 (16.1%)	27 (87.1%)
	Subtotal (N=98)	17 (17.3%)	15 (15.3%)	70 (71.4%)
After 3 h of labor	Picker	0 (0.0%)	0 (0.0%)	6 (15.8%)
	Sorter	4 (14.8%)	4 (14.8%)	23 (85.2%)
	Packer	4 (17.4%)	5 (21.7%)	22 (95.7%)
	Subtotal (N=88)	8 (9.1%)	9 (10.2%)	51 (58.0%)
Total (N=186)		25 (13.4%)	24 (12.9%)	121 (65.1%)

[†]Percentage of positive samples based on samples analyzed. [††]Positive samples by PCR.

68.3 percentage of samples, respectively). Johnston et al. (2005) coincide with our study since they also found an increase in coliforms levels from harvest through packing, with an increase occurring also at the rinse step.

Only 3% of the fresh produce was positive for *E. coli* (Table 2). Fresh produce sampled at the field (plant, harvest and field containers) contained 1.06% of *E. coli*, and the produce collected at the packinghouse had 4.0% being the waxing operation one of the most contaminated (Table 3). The levels of *E. coli* increased at the uploading ramps in the packinghouse with a range of 0.3 log to 1.6 log CFU/100g. *Salmonella* spp. levels remained consistently low with 2.6% of the positive samples among the harvest, field container, unloading ramp, sorting, and packing bins (Table 3). Unlike *E. coli*, *Salmonella*-positive samples were distributed at the field and only a few on the packinghouse points. The results of this study coincide with that of Mukherjee et al. (2004) that reported numbers of 2.3% of produce contaminated with *Salmonella*, however, some studies such as Gallegos-Robles et al. (2008) have reported higher levels (37%) of bell pepper produces contaminated with *Salmonella*. One drawback is the fact that both studies used a smaller amount of samples as the one reported in this study, thus large number of samples need to be sampled to determine the behavior of the pathogen.

A total of 186 worker hands were analyzed as follows: 98 samples after workers washed their hands before labor started and 88 samples were taken after 3 h of labor (Table 4). The results showed the presence of 15.3 and 71.4% of *E. coli* and coliforms before labor, respectively. The levels of *E. coli* and coliforms after washing hands were 1.76±0.73 and 2.77±0.94 log, respectively. Copper sulfate was used at the field as a hand washing disinfectant solution to reduce the presence of microorganisms from hands, however, *E. coli* and coliforms remained present after washing hands. This can be related to inadequate hygienic practices, including the use of contaminated water, lack of or insufficient sinks and manual faucets infrastructure. According to Montville et al. (2002) it is imperative to apply an efficient technique for washing hands before starting working with fruits and vegetables. This technique may include a continuous training by the food safety staff (FDA, 1998). A hand washing technique recommended by Jimenez et al. (2007) consist in washing hands with an antibacterial soap for 30 s, rinsing with water for 15 s, drying with paper towels and rubbing with an alcohol-based gel to reduce at least 3.5 log of *Salmonella* on hands. On the contrary, contamination during the first 3 h of work showed 10.2 and 58% of contaminated hands with *E. coli* and coliforms, respectively. This may be due to the transfer of bacteria through direct contact with contaminated surfaces and fresh produce. Additionally, around 20% of worker hands before starting to work were contaminated with coliforms with a concentration of 1.8±0.9 log CFU/hands but showed no coliforms after 3h of work; while 53% were contaminated with coliforms at a higher concentration 3.0±1.0 log CFU/hands and the contamination continued after 3 h of labor (data not shown). This may be due to the carryover of the bacteria from hands to workers, fresh produce, or surface contact areas. Additionally, the residual bactericidal effect of copper sulfate applied during hand washing before starting the labor can also be related to the absence of coliforms after 3 h of labor.

The presence of *Salmonella* spp. was detected in 17.3% of worker hands before starting to work, including hands of pickers, sorters and packers (Table 4). After 3 h of work, only 9.1% of the sample hands were contaminated; the pickers hands were not included (Table 4). *Salmonella* showed a slightly increase throughout postharvest handling, suggesting a potential transfer of the pathogen from contaminated hands to

Table 5. Presence of *Salmonella* spp., *Escherichia coli* and coliforms on contact surface areas of packinghouse equipment.

Surface contact	No. of positive samples (%)†		
	***Salmonella* spp.††**	***Escherichia coli***	**Coliforms**
Unloading ramp	0 (0.0%)	3 (9.1%)	29 (87.9%)
Roller	0 (0.0%)	2 (6.1%)	17 (51.5%)
Conveyor belts	3 (9.1%)	0 (0.0%)	21 (63.6%)
Packing bin	2 (6.1%)	1 (3.0%)	25 (75.8%)
Total (N=132)	5 (3.8%)	6 (4.5%)	92 (69.7%)

†Percentage of positive samples based on samples analyzed; ††Positive samples by PCR.

fresh produce. Jimenez et al. (2007) reported that about 0.21% of the bacteria are transferred from contaminated hands to pepper produce. *S. enterica* serovar Typhimurium was confirmed by serotyping in 50 of the isolated strains.

Microbial contamination on surface contact areas, including *E. coli* and coliforms increased from harvest throughout packing. *E. coli* and coliforms were found in 4.5 and 69.7% of the total surface contact areas analyzed, respectively (Table 4). The levels of contamination ranged from 1.45±1.15 log CFU/400cm^2 and 1.23±0.83 log CFU/400cm^2 for *E. coli* and coliforms, respectively. The highest frequency of *E. coli* (9.1%) and coliforms (87.9%) was found in the unloading ramp with concentrations of 2.05±1.02 and 1.63±0.98 log CFU/600cm^2, respectively. *E. coli* and coliforms were constant from unloading ramp through the boxes ready for distribution. *Salmonella* spp. was present in 9.1 and 6.1% of conveyor belts and packing bins sample (Table 5). Nine of the isolated strains were confirmed as *S. enterica* serovar Typhimurium.

Duffy et al. (2005) also found the presence of *Salmonella* and *E. coli* in unloading ramps and conveyor rollers at fresh produce packinghouse in Texas. Additionally, Montville and Shaffner (2003) demonstrated the transfer of bacteria from surface contact areas to lettuce (*Lactuca sativa* L.). The findings of the present study suggest that surface contact areas are an important source of microbiological contamination of bell pepper produce in the packinghouse.

Pathogenic *E. coli* was not detected in any of the 900 samples tested. However, *S. enterica* serovar Typhimurium was confirmed in worker hands and surface contact areas. *S. enterica* serovar Typhimurium is one of the most frequently isolated strains worldwide, specifically in Mexico (Gutiérrez -Cogco et al., 2000; Wasyl et al., 2006). López-Cuevas et al. (2009) found *Salmonella* Typhimurium, as well as Infantis, Anatum, Agona, Oranienburg, Minnesota in agricultural water in the Culiacan region. Similarly, Estrada-Acosta et al. (2014) found *Salmonella* Oranienburg in the Culiacan river. These findings highlight the needs for microbial determination numbers to determine the impact of the pathogen in water. The presence of *S. enterica* serovar Typhimurium in worker hands and contact surface areas eventually causes illness to consumers. Infections and outbreaks with *S. enterica* serovar Typhimurium have been linked to consumption of contaminated food such as vegetable salads (Doré et al., 2004; Wasyl et al., 2006).

Conclusions

Microbiological contamination of bell pepper produce gradually increased during handling in the production system from the time fruit is harvested in the field to its packaging process, being the surface contact areas the main vehicles of transmission of microorganisms to the produce. This study shows that water used for washing hands, worker hands, and surface contact areas were the main source of contamination. Procedures such as good agricultural practices, good Manufacturing practices, and HACCP can minimize but not eliminate foodborne pathogens in agricultural products. Thus, the study demonstrates the presence of contamination that suggests the need for both the improvement of the good agricultural practices and the evaluation of alternative disinfectants. Further studies including a larger number of participating farms assessing the presence and persistence of other bacterial indicators and virulence, as well as fingerprints data of each of the microorganism detected to determine the relationship and source of contamination are needed.

Conflict of interests

The authors did not declare any conflict of interest.

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