PATHOGEN DETECTION

DEVELOPMENT OF A DNA MICROARRAY CHIP FOR IDENTIFICATION OF *LISTERIA* SPECIES, PARTIAL SEROTYPING AND ASSESSMENT OF THE VIRULENCE POTENTIAL OF *LISTERIA MONOCYTOGENES* ISOLATES

(L. Ma, G. Zhang, and M. P. Doyle)

Listeria monocytogenes is a leading cause of death attributed to foodborne bacterial pathogens. Lack of sufficient scientific information has led regulatory agencies to consider any strain of *L. monocytogenes* to be potentially pathogenic for humans although several lines of evidence indicate there are differences in virulence potential among *L. monocytogenes* isolates. Our ultimate goal is to develop a DNA microarray chip that can differentiate and identify *Listeria* species, including *L. monocytogenes,* as well as subtype and assess the virulence potential of *L. monocytogenes* isolates. Our initial approach to developing this chip was to design a set of 128 probes targeting 16S rRNA and *iap* genes of *Listeria* spp. (for differentiation and identification of *Listeria* spp.), and the *iap*, *gltA-gltB*, and *inlB* genes of *L. monocytogenes* (for partially serotyping and assessing the virulence potential of *L. monocytogenes* isolates). The probes (17- to 37-mer) were designed through the following steps: retrieving all available related gene sequences from GenBank; alignment of retrieved sequences using Clustral-X version 1.80; manual editing of alignment by SeaView; identification of regions for probe design; and design of specific probes using probe design software Sarani. For every probe, a control probe containing one central mismatch was included for accurate discrimination between true signal and random hybridization. The probes were synthesized with 5' or 3' amine modification for covalent attachment to substrate slides and several probes were synthesized with additional 12-mer spacers for studying the effect of general steric hindrance. Construction of the microarray chip is underway using an OmniGrid Accent Microarrayer and the functionality of the chip will be validated through serial hybridization with genomic DNAs from a collection of *Listeria* strains. The developed DNA microarray chip will enable rapid and accurate discrimination among six *Listeria* species and partial serotyping, and assess the virulence potential of *L. monocytogenes* isolates, such as those of food origin. With addition of more probes that specifically target virulence-associated genetic markers, the chip should be useful for rapid and more accurate global assessment of the virulence potential of any *L. monocytogenes* isolate. This DNA chip can be used for large-scale population genetic analysis of *L. monocytogenes* isolates and information gained through such studies would be the scientific basis for differentiating highly virulent from less virulent or avirulent strains of *Listeria.* This would provide an assay to enable food processors, regulatory agencies, and public health organizations to differentiate significant public health concerns such as food contaminated with a highly virulent strain of *L. monocytogenes* in contrast to an unimportant avirulent *L. monocytogenes* food contaminant. In addition, with resolution beyond the species level, the chip may be a useful subtyping tool for tracking contamination sources in the food-processing environment.

A SOLID AGAR OVERLAY METHOD FOR RECOVERY OF HEAT-INJURED *LISTERIA MONOCYTOGENES*

(Z. Yan, J. Gurtler, and J. L. Kornacki)

A solid agar overlay method was developed for recovery of heat-injured *Listeria monocytogenes*. A pre-solidified non-selective medium, tryptic soy agar with 0.6% yeast extract (TSAYE, 2% agar), was aseptically overlaid onto the top of a solidified selective medium; modified oxford agar (MOX). In principle, injured bacterial cells are resuscitated on the TSAYE overlay before diffusion of selective agents from MOX can inhibit their recovery. A five-strain cocktail of *L. monocytogenes* was heat injured by subjecting the cells to 58°C for 6 min in a water-jacketed flask filled with TSB broth. Both freshly grown and heat-treated cells of *L. monocytogenes* were plated onto TSAYE, MOX and TSAYE/MOX overlaid plates. No significant differences ($P < 0.05$) were found among the three media for recovery of freshly grown (e.g., uninjured) bacterial cells. Selective medium MOX recovered significantly ($P < 0.05$) less *L. monocytogenes* cells than on non-selective medium TSAYE and the TSAYE/MOX overlaid plates. In contrast, there were no significant differences among the TSAYE and TSAYE/MOX overlaid agar plates prepared 0, 2, 4, 6, 8, 16, and 24 h prior to plating heat-injured bacterial cells. TSAYE/MOX overlaid agar was able to differentiate *L. monocytogenes* from a mixture of three additional

foodborne pathogens; *Salmonella* spp, *E. coli* O157:H7 and *Yersinia enterocolitica*. This solid agar overlay method for recovery of heat-injured *L. monocytogenes* cells is less time-consuming and less complicated than the conventional overlay/underlay technique and reported thin agar layer overlay methods.

COMPARISON OF USDA CULTURE PROCEDURE AND POLYMERASE CHAIN REACTION (PCR) DETECTION OF *LISTERIA MONOCYTOGENES* ON DELI MEATS

(C.-M. Lin, L. Zhang, and M. P. Doyle)

A polymerase chain reaction (PCR) assay (BAX®, DuPont, Wilmington, DE) for detecting *Listeria monocytogenes* in meat products has been adopted by the US Department of Agriculture (USDA). In addition, USDA-FSIS has promoted using a large sampling size such as an entire package for detecting *L. monocytogenes* in meat products. This study was conducted to compare the BAX-PCR assay and the USDA culture enrichment method to detect *L. monocytogenes* on deli meats contaminated during slicing by a slicer blade inoculated with listeriae. Two types of deli meat, salami and bologna, were tested. The salami had a low pH and moisture content and the bologna contained antimicrobials to *L. monocytogenes,* hence neither meat would support the growth of listeriae. A five-strain (Bil Mar, Coleslaw, Plantation, Jalisco, OM) mixture of *L. monocytogenes* at 10^2 cfu was inoculated onto the surface of the blade of a commercial slicer. A commercial log of meat product was sliced in its entirety and five slices per package were packed, vacuum-sealed, stored at 4°C, then sampled at 1, 30, 60, and 90 days post-slicing. Meat sliced before the blade was inoculated with *L. monocytogenes* was used as the negative control. Meat from eight negative-control packages was inoculated with 10^1 or 10^2 cfu of *L. monocytogenes* to serve as positive controls. Studies with each type of meat were done in duplicate. A total of 132 samples were analyzed at each sampling date. The entire package of deli meat was stomached with UVM broth and incubated at 30°C for 24 h. For the USDA culture enrichment procedure, 0.1 ml of UVM enrichment culture was transferred into Fraser broth then incubated and streaked onto modified Oxford agar (MOX) plates, and a loopful of the enrichment culture was streaked directly onto MOX plates. Presumptive isolates of *L. monocytogenes* obtained from either culturing in Fraser broth or by direct plating onto MOX were confirmed by hemolysis on horse blood agar plates, then biochemical assay with API strips (bioMériux Inc., Hazelwood, MO) and BAX-PCR. For the BAX-PCR assay, 0.1 ml of the UVM-enrichment culture was transferred into BAX-*Listeria* enrichment broth. PCR-positive samples were further assayed by streaking the BAX-*Listeria* broth onto MOX and horse blood agar plates, then isolates were confirmed with API strips. A three-tube most probable number (MPN) method was used to enumerate *L. monocytogenes* populations in positive samples. There were very few *Listeria*-positive samples. For the two trials for each type of meat, at most two meat packages were confirmed *L. monocytogenes*-positive on the first sampling day. The number of *Listeria*-positive samples declined during storage. No sample was confirmed *Listeria*-positive at 30 and 60 days for either salami or bologna; however, one 90-day sample of salami in the second trial was confirmed *L. monocytogenes*-positive. None of the negative controls was positive and *L. monocytogenes* in the positive controls inoculated at the 10^1 cfu level could not be recovered at 30 and 60 days on either salami or bologna. Based on MPN, *L. monocytogenes* populations were very low (from < 0.06 to 0.75 MPN/g) and decreased gradually during storage, even in positive control samples. The number of *L. monocytogenes*-positive samples determined by PCR or culture enrichment was similar. Most of the *Listeria*-positive samples were detected by both PCR and culture enrichment; however, some samples were determined *Listeria*-positive by PCR but not by culture enrichment and vice versa. Our results indicate that the ability of the BAX-PCR assay and the USDA culture enrichment to detect small cell numbers of *L. monocytogenes* on salami and bologna was similar, and that neither bologna with antimicrobial agents nor salami supported the growth of *L. monocytogenes* throughout 90 days of storage at 4ºC.

DIRECT MICROSCOPIC OBSERVATION AND VIABILITY DETERMINATION OF *CAMPYLOBACTER JEJUNI* ON CHICKEN SKIN

(W. Chantarapanont, M. Berrang, and J. F. Frank)

The objective of this study was to develop a method to determine survival of *Campylobacter jejuni* at specific sites on chicken skin and to use this method to observe survival of *C. jejuni* at various locations on the skin during storage. This method employs confocal laser scanning laser microscopy (CSLM) to visualize *Campylobacter jejuni* transformed with a green fluorescent protein plasmid (GFP-*Campylobacter*) and stained with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). The green fluorescence of *C. jejuni* cells and the red fluorescent CTC-formazan in

viable *Campylobacter* cells were clearly visible on chicken skin. GFP-*Campylobacter* remaining on the chicken skin surface after rinsing were mostly located in crevices, entrapped inside feather follicles with water, and entrapped in the surface water layer. Most viable cells were entrapped with water in the skin crevices and feather follicles. These sites provide a suitable microenvironment for GFP-*Campylobacter* to survive. The population of *C. jejuni* on chicken skin decreased by 1 $\log_{10}$ unit during storage at 25°C for 24 h. *Campylobacter jejuni* located in sites 20-30 µm beneath the chicken skin surface maintained viability during incubation at 25°C. *C. jejuni* on chicken skin stored at 4°C maintained constant numbers during 72 h of incubation with no significant changes in population of feather follicles or crevices. Live and dead cells were initially retained with water on the skin and penetrated into skin follicles and channels during storage. Microscopic observations of GFP-producing cells allowed identification of survival niches for *C. jejuni* present on chicken skin.

APPLICATION OF NESTED PCR TO DETECTION OF *SALMONELLA* IN POULTRY ENVIRONMENT

(J. J. Maurer)

Isolation of *Salmonella* from poultry environmental and processing plant samples requires sampling large numbers of areas within the poultry house or plant. Subsequently, this results in a large volume of work for a microbiology laboratory especially when the protocol requires inclusion of a delayed secondary enrichment for isolation of *Salmonella*. This study examined the use of PCR to identify those secondary enrichments containing *Salmonella*. The unique *Salmonella* virulence gene *invA* was chosen as the target for development of a nested-PCR because of its uniform distribution among *Salmonella* serotypes. The use of nested-PCR primers increased sensitivity of detection 100-fold, resulting in detection of as few as 4 cells. There was a statistically significant, strong positive correlation between PCR and culture results as determined by chi-squared test ($p < 0.001$) and kappa test (0.915; excellent agreement). Using PCR as a screen of primary enrichments for presumptive *Salmonella* contamination, we improved our efficiency at isolating *Salmonella* upon secondary enrichment by 20 % and no false negatives were observed. This method will not only validate the use of secondary enrichment procedures, but also reduce costs and manpower for surveillance of *Salmonella*.

DETECTION OF *SALMONELLA* AND *CAMPYLOBACTER* IN POULTRY BY PCR-ELISA

(J. J. Maurer)

Contamination of retail poultry by *Campylobacter spp.* and *Salmonella enterica* is a significant source of human diarrheal disease. Isolation and identification of these microorganisms requires a series of biochemical and serological tests. In this study, *Campylobacter ceuE* and *Salmonella invA* genes were used to design probes in PCR-ELISA, as an alternative to conventional bacteriological methodology, for the rapid detection of *Campylobacter jejuni*, *Campylobacter coli*, and *Salmonella enterica* from poultry samples. With PCR-ELISA (40 cycles), the detection limit for *Salmonella* and *Campylobacter* was 2×10^2 cfu/ml and 4×10^1 cfu/ml, respectively. ELISA increased the sensitivity of the conventional PCR method by 100- to 1,000-fold. DNA was extracted from carcass rinses and tetrathionate enrichments and used in PCR-ELISA for the detection of *Campylobacter* and *S. enterica*, respectively. With PCR-ELISA, *Salmonella* was detected in 20 of 120 (17%) chicken carcass rinses examined, without the inclusion of an enrichment step. Significant correlation was observed between PCR-ELISA and cultural methods (kappa = 0.83; chi-squared test: $p < 0.001$) with only one false negative (1.67%) and four false positives (6.67%) when PCR-ELISA was used to screen sixty tetrathionate enrichment cultures for *Salmonella*. With PCR-ELISA, we observed positive correlation between the ELISA absorbance (Optical Density_{405nm}) and *Campylobacter* cell number in carcass rinse, as determined using standard culture methods. Overall, PCR-ELISA is a rapid and cost-effective approach for the detection and enumeration of *Salmonella* and *Campylobacter* on poultry.

A RESTRICTION FRAGMENT LENGTH POLYMORPHISM BASED POLYMERASE CHAIN REACTION AS AN ALTERNATIVE TO SEROTYPING FOR IDENTIFYING *SALMONELLA* SEROTYPES

(J. J. Maurer)

The phase 1 (*fliC*) and phase 2 (*fljB*) *Salmonella* flagella genes were analyzed by RFLP-PCR to aid in the identification of different *Salmonella* serotypes. Twenty-four phase 1 flagellin and eight phase 2 flagellin genes could be differentiated among each other using restriction endonucleases *Sau*3A and *Hha*I in RFLP-PCR analysis. These flagellin genes comprise the major antigenic formulas for fifty-two serotypes of *Salmonella* spp., which

include the common serotypes found in poultry and other important food animal species. With the knowledge of the O antigen composition determined from conventional O serotyping, ninety percent of the *Salmonella* serotypes could be identified using this double restriction enzyme RFLP analysis of *fliC* and *fljB* genes. This RFLP-PCR flagellar typing scheme was successfully applied to the identification of serotype for 112 *Salmonella* isolates obtained from poultry environments. There was a significant correlation between RFLP PCR and conventional serotyping (chi-square $p < 0.001$). Overall, PCR-RFLP proved to be a fast, accurate and economic alternative approach to serotyping *Salmonella* spp. such that it is now part of diagnostic services offered to the poultry industry for identifying *Salmonella* serotypes.

RAPID DETECTION OF POULTRY *SALMONELLA ENTERICA* SEROTYPES ENTERITIDIS, HADAR, HEIDELBERG, AND TYPHIMURIUM BY MULTIPLEX PCR TO COMMON O AND H ANTIGEN GENES AND ALLELES

(J. J. Maurer)

There are over 2,000 different *Salmonella* serotypes but few are commonly associated with human disease. Currently, serotyping relies on a time consuming process that requires implementing a battery of antisera for typing O and H antigens of *Salmonella enterica*. A rapid multiplex PCR-based molecular serotyping scheme was developed with an ability to identify *S. enterica* serotypes Enteritidis, Hadar, Heidelberg, and Typhimurium isolated from the poultry environment. The nucleotide sequence of the O-antigen biosynthesis *rfb* operons for *Salmonella* O serogroups D1, B, C1, C2, and E1 were analyzed to identify sequences suitable for designing multiplex primer sets. By analyzing the highly variable nucleotide sequences in the central region of the phase-1 and phase-2 *Salmonella* flagella genes *fliC* and *fljB*, multiplex PCR's were designed to identify the *fliC* genes associated with the phase 1 antigen types i, g,m r, z_{10} and the *fljB* genes belonging to two major phase 2 antigen complexes, 1,2, 1,5, 1,6, 1,7 and e,n,x, e,n,z_{15}. These antigens constitute the antigenic formulas for common clinical and poultry *S. enterica* serotypes. The multiplex PCR conditions were optimized and discrimination was achieved, identifying O or H antigen gene(s) and alleles based upon the unique sizes of the amplicons from multiplex PCR. The serotyping, multiplex PCR was comparable to the standard serological typing methods for correctly identifying *S. enterica* serotype (kappa = 1.00; chi-squared test: $p < 0.001$). Coupled with primary enrichment using tetrathionate brilliant green broth, multiplex PCR correctly identified poultry samples contaminated with *S. enterica* Enteritidis, Kentucky, and Typhimurium. These multiplex PCR assays for detecting specific O and H antigen gene alleles can become a rapid and cost-effective alternative approach to serotyping for the identification of common poultry *Salmonella* serotypes. In fact, this multiplex PCR has now become part of diagnostic services offered to poultry industry for identifying *Salmonella* serotypes.

INOCULATION STRATEGIES AND PARASITE RECOVERIES FROM EXPERIMENTALLY SPIKED PRODUCE

(C. Tatum and Y. R. Ortega).

Protozoan parasites have long been associated with water and foodborne outbreaks. Those parasites that most commonly have been associated with foodborne outbreaks, causing prolonged diarrheal illness, include *Cryptosporidium parvum, Cyclospora cayetanensis*, and *Giardia lamblia.* Most parasites need a host to multiply, therefore conventional propagation used with bacterial pathogens cannot be applied. Due to this limitation, recovery procedures are very crucial for parasite identification.

To evaluate recovery procedures, experimental spiking of basil, lettuce, and raspberries were done using three inoculation methods (spot, spray, and dip) and various concentrations of the three parasites (10^2 – 10^5 oocysts/produce). The greatest recoveries were obtained through spot inoculation followed by spray and dip inoculations. In all three food matrices *Giardia* cysts (20 – 90%) were recovered to a greater extent than *Cryptosporidium* while *Cyclospora* had the lowest recoveries. Recoveries of *Cryptosporidium* and *Giardia* were greatest on basil and lettuce than raspberries.

DEVELOPMENT OF A METHOD TO QUANTIFY EXTRACELLULAR CARBOHYDRATE COMPLEXES PRODUCED BY *ESCHERICHIA COLI* O157:H7

(J.-H. Ryu and L. R. Beuchat)

Escherichia coli O157:H7 is known to produce exopolysaccharides (EPS). Production on the surface of foods and food processing equipment may result in enhanced protection against various environmental stresses. EPS may

also be involved in cell/cell recognition and interaction, biofilm formation, and adhesion. Biofilm formation and adhesion are extremely important because cells become more resistant to removal from foods and food-contact surfaces in processing plants and to inactivation by sanitizers. There is evidence that EPS is involved in the development of surface film, adhesion of cells, and the formation of a complex three-dimensional biofilm structure. Since EPS may be involved in many steps of biofilm formation and, in fact, be a component of biofilm, it is important to use a standard method to quantify the amount of EPS produced under various environmental conditions in order to more easily develop intervention strategies to prevent or eliminate biofilm development.

Bacterial surface polysaccharides can be categorized in two general groups, those bound to the cell surface by attachment to lipid A, i.e., lipopolysaccharides (LPS), and those associated with the cell surface as a capsule and extracellular slime, EPS. It is difficult to separate LPS from EPS according to their presence, functions, and roles in biofilm formation. It may be difficult to make a complete physical separation of the two polymers because components of the microbial cell wall undergo constant turnover and may be excreted and lost from the cell surface as the cell ages or is subjected in natural or laboratory environments to high shear forces. Exopolysaccharides are probably present with other carbohydrates as carbohydrate complexes. Other carbohydrates may include mono- or oligosaccharides secreted by the cell or cleaved from the EPS physically or enzymatically, and polysaccharides derived from cell surface components. Consequently, the effects of EPS on cell physiology and behavior in response to external factors should be considered in broader terms of extracellular carbohydrate complexes (ECC). We defined ECC as those carbohydrates secreted from cells or loosely attached to the cell surface which can be detached by heat treatment. ECC might include EPS (slime and capsule) and other carbohydrates such as components of LPS, cell walls, and degraded EPS.

We undertook a study with the objective of optimizing a procedure to separate ECC fractions I and II present in EPS formed by *E. coli* O17:H7 and to standardize a method to quantify these complexes on a per cell basis. The ultimate goal is to develop a standard procedure to examine the production of ECC by *E. coli* O157:H7 in biofilms formed on food contact surfaces in processing plants and on foods as affected by conditions to which they are exposed. ECC fraction I was removed from *E. coli* O157:H7 cells produced on tryptic soy agar and lettuce juice agar by centrifugation. To remove ECC fraction II, cells were heated at 100°C for 10 min, then centrifuged. The sum of ECC fraction I and II was considered as the total ECC produced by *E. coli* O157:H7. A correlation between cell mass and turbidity (OD_{750nm}) of cell suspensions was determined. Cell mass has a linear relationship (R^2 = 0.93) with turbidity of cell suspensions from which ECC is removed. The amount of ECC produced on a per cell basis was calculated by dividing total amount of ECC (µg/ml) produced by the turbidity (OD_{750nm}) of heated cell suspension after removal ECC fractions I and II. We succeeded in developing a method for separating ECC from cells of *E. coli* O17:H7 and conditions have been optimized. A standard method to estimate the amount of ECC produced on a per cell basis was also developed. Using these procedures to prepare extract of ECC from *E. coli* O157:H7 and standardize values, production of ECC on a per cell basis can be estimated and a comparison of the amount of ECC produced by the pathogen grown under different environmental conditions can be accurately measured.

Survival and Recovery of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on Lettuce and Parsley as Affected by Method of Inoculation, Time Between Inoculation and Analysis, and Treatment with Chlorinated Water

(M. M. Lang, L. J. Harris, and L. R. Beuchat)

Given sufficient time and appropriate environmental conditions after contamination, pathogens can grow to populations exceeding 10^7 cfu/g of lettuce and 10^6 cfu/g of parsley. Conditions that result in contamination of produce with pathogens and subsequent growth during storage have been described. A wide range of chemical sanitizers and physical treatments for decontamination of fresh produce has been evaluated. Results are difficult to compare, however, because of the numerous variations in methodologies. The lack of uniformity of methods used to treat produce with sanitizers and enumerate microorganisms surviving treatments makes it difficult to assess their effectiveness and establish industry recommendations and guidelines for their use. The development of a standard method would minimize or eliminate variations in methodologies used in various laboratories, thereby enabling a comparison of pathogen reductions resulting from treatment with various sanitizers. A single method may not be applicable for all fruits and vegetables but a basic test method that could be modified as necessary to accommodate natural variations in fresh and fresh-cut produce would be the goal.

One of the objectives of the study reported here was to evaluate three methods (dip, spot, and spray) for inoculating *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* onto the surface of two types of leafy salad vegetables, lettuce and parsley, with the goal of establishing a procedure for use in a standard method to test the efficacy of sanitizers. A second objective was to examine the effect of time between application of inoculum and analysis of lettuce and parsley on the viability and recovery of pathogens. Inocula applied to lettuce and parsley were subjected to two drying times, followed by either no treatment or treatment with water or chlorine then analyzed for the number of surviving cells. Inocula were dried for 2 h at 22°C or for 2 h at 22°C followed by 22 h at 4°C before treating with water (control) or chlorine (200 µg/ml). Significantly ($\alpha = 0.05$) higher populations (cfu/lettuce or parsley sample) of *E. coli* O157:H7 and *Salmonella* were recovered from dip-inoculated produce compared to spot- or spray-inoculated produce. This is attributed to larger numbers of cells adhering to lettuce and parsley subjected to dip inoculation. Populations of *E. coli* O157:H7 and *Salmonella* recovered from lettuce inoculated by spot and spray methods were not significantly different but populations recovered from spot-inoculated parsley were significantly higher than those recovered from spray-inoculated parsley, even though the number of cells applied was the same. Significantly different populations of *L. monocytogenes* were recovered from inoculated lettuce (dip > spray > spot); populations recovered from dip-inoculated parsley were significantly higher than those recovered from spot- or spray-inoculated parsley, which were not significantly different from each other. Populations of pathogens recovered from lettuce and parsley after drying inoculum for 2 h at 22°C were significantly higher than or equal to populations recovered after drying for 2 h at 22°C followed by 22 h at 4°C. Significant differences (water > chlorine) were observed in populations of all pathogens recovered from treated lettuce and parsley, regardless of inoculation method and drying time. It is recommended that spot inoculation with a drying time of 2 h at 22°C followed by 22 h at 4°C be used to determine the efficacy of chlorine and other sanitizers in killing foodborne pathogens on lettuce and parsley.

INFLUENCE OF VARIATIONS IN METHODOLOGY ON POPULATIONS OF *LISTERIA MONOCYTOGENES* RECOVERED FROM LETTUCE TREATED WITH SANITIZERS

(A. B. Burnett, M. H. Iturriaga, E. F. Escartin, C. A. Pettigrew, and L. R. Beuchat)

The lack of a standard method(s) to quantitate pathogens on raw fruits and vegetables has resulted in great variations in methodology used by researchers and in commercial testing laboratories. While some of these variations may not affect the efficiency of enumerating pathogens on a given produce item, most have not been properly evaluated or validated. The importance of optimum procedures for recovering pathogens that may be stressed or injured as a result of desiccation or exposure to chemical sanitizers, for example, is increased when the efficacy of decontamination treatments is being assessed. If the enumeration method does not recover all viable cells from treated produce, an underestimation of populations will result.

Progress is being made in developing and validating a standard method to evaluate the effectiveness of produce sanitizers, although modifications of a basic method will likely be necessary to achieve maximum recovery of pathogens surviving treatment. Major variations in sanitizer efficacy methodology currently used by researchers include inoculation of produce by dipping in a cell suspension versus spot inoculation of the test pathogen, drying inoculum for times ranging from a few seconds to 24 h, failure to use a neutralizing agent to terminate the activity of the lethal component, and rinsing, stomaching, or blending to process treated samples for enrichment or direct plating. In only a few studies have comparisons of variations in specific steps in produce sanitizer efficacy methods been made. Survival of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on tomatoes, as affected by drying time after application of inoculum, and subsequent effectiveness of point-of-use sanitizers in reducing populations have been investigated. We have examined sample processing methods for their influence on populations of *Salmonella* recovered from twenty-six types of raw fruits, vegetables, and herbs. Overall, processing samples by washing in 0.1% peptone, stomaching, or homogenizing did not substantially affect the number of *Salmonella* recovered.

The study reported here was done to determine if variations in methodology influence the efficacy of chlorinated (200 µg/ml free chlorine) water and FIT® Professional Line Produce Cleaner (FIT®) in killing *L. monocytogenes* spot inoculated onto iceberg lettuce. Variations in methodology included composition of broth media used to grow cells to prepare inocula, number of strains present in the inoculum, time and temperature between inoculation and treatment with sanitizers, sample processing method, and composition of direct plating media used to enumerate the pathogen. The efficacy of the two sanitizers was not influenced by the composition of the medium used to culture *L. monocytogenes* used in inocula, the number of strains in the inoculum, or the

recovery medium used to enumerate the pathogen on lettuce after treatment. Drying inoculum on lettuce for 45 min at 37°C caused more cells to die or not be retrieved compared to drying inoculum for 30 min at 25°C. However, the percentage of cells in the inoculum recovered from lettuce treated with chlorine or FIT® was not significantly different, regardless of the drying method. Stomaching, homogenizing, or stomaching followed by homogenizing lettuce treated with sanitizers resulted in recovery of similar numbers of *L. monocytogenes*, indicating that stomaching and homogenizing are equivalent in extracting cells; the sequential use of both processing methods did not substantially increase the efficiency of recovery. Washing lettuce with water or treating lettuce with 200 µg/ml chlorine or FIT® resulted in decreases in populations of 0.60, 1.76, and 1.51 $\log_{10}$ cfu/lettuce, respectively, regardless of variations in test parameters. Reductions caused by sanitizers were significantly greater ($\alpha = 0.05$) than that observed for water but not significantly different from each other. It is concluded that evaluation of sanitizers for their efficacy in killing *L. monocytogenes* on lettuce can be determined by spot inoculating 50 µl of a five-strain mixture of cells from 24-h cultures suspended in 5% horse serum albumen, followed by drying the inoculum for 45 min at 37°C, treatment by submerging in 50 ml of sanitizer for 5 min, stomaching samples in 50 ml of Dey-Engley neutralizing broth for 2 min, and enumerating survivors on modified Oxford medium.

EVALUATION OF INOCULATION METHOD AND INOCULUM DRYING TIME FOR THEIR EFFECTS ON SURVIVAL AND EFFICIENCY OF RECOVERY OF *ESCHERICHIA COLI* O157:H7, *SALMONELLA*, AND *LISTERIA MONOCYTOGENES* INOCULATED ON THE SURFACE OF TOMATOES

(M. M. Lang, L. J. Harris, and L. R. Beuchat)

Contamination of raw produce with pathogenic microorganisms can occur at any of several points from the field through the time of consumption. Given sufficient time and appropriate environmental conditions, pathogens can grow to populations exceeding 10^7 cfu/g of tomato. Work has been done to define conditions that result in contamination of produce and subsequent growth of pathogens during storage. Researchers have also evaluated the effectiveness of a wide range of chemical sanitizers and physical treatments to decontaminate fresh produce. Results of studies done in different laboratories are difficult to compare, however, because of numerous variations in methodologies employed and incompleteness in describing results. A study was undertaken with the objective to evaluate procedures for inoculating *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* onto the surface of tomatoes with the goal of selecting an inoculation procedure to be used in a standard method. Dip, spot, and spray inoculation were evaluated. A second objective was to examine the effect of time between application of inoculum and analysis of tomatoes on the viability and retrievability of pathogens. Inocula applied to tomatoes were subjected to two drying times followed by either no treatment or treatment with water (control) or chlorine (200 µg/ml), then analyzed for the presence of surviving cells.

Five-strain mixtures of *Escherichia coli* O157:H7, *Salmonella*, or *Listeria monocytogenes* were applied to tomatoes by dip, spot, or spray inoculation methods. Inocula were dried for 1 or 24 h at 22°C before tomatoes were treated with water (control) or chlorine (200 µg/ml). Significantly ($\alpha = 0.05$) higher populations (cfu/tomato) of *E. coli* O157:H7 and *Salmonella* were recovered from dip-inoculated tomatoes compared to spot- or spray-inoculated tomatoes. This is attributed to larger numbers of cells adhering to tomatoes subjected to dip inoculation. Populations of *E. coli* O157:H7 and *Salmonella* recovered from spot- and spray-inoculated tomatoes containing the same initial number of cells were not significantly different. Significantly different populations of *L. monocytogenes* were recovered from inoculated tomatoes (dip > spot > spray). Populations of pathogens recovered from tomatoes were significantly higher when inocula were dried for 1 h compared to 24 h. Significant differences (water > chlorine) were observed in populations of all pathogens recovered from tomatoes treated with chlorine, regardless of inoculation method or drying time. Results indicate that inoculation method, drying time, and treatment affect survival and/or recovery of foodborne pathogens inoculated onto the surface of tomatoes. It is recommended that spot inoculation with a drying time of 24 h at 22°C be used in a standard method to determine the efficacy of chlorine and other sanitizers in killing foodborne pathogens on tomatoes.

EFFECT OF INOCULUM SIZE, RELATIVE HUMIDITY, STORAGE TEMPERATURE, AND RIPENING STAGE ON THE ATTACHMENT OF *SALMONELLA* MONTEVIDEO TO TOMATOES AND TOMATILLOS

(M. H. Iturriaga, E. F. Escartin, L. R. Beuchat, and R. Martinez-Peniche)

Bacterial survival and growth on and in fruits and vegetables depends, in part, on their ability to attach to surfaces. Sessile microorganisms have advantages in that they are more difficult to mechanically remove from

surfaces and are more resistant to disinfectants compared with planktonic cells. It has been observed that attachment of bacterial cells is affected by several factors, including the medium in which they are grown, motility, temperature, length of contact time, and production of extracellular polysaccharides. Information describing the attachment of human pathogens on fruits and vegetables is limited; however, the process appears to be similar to that of adhesion of plant pathogenic and non-pathogenic bacteria on leaves. One possible intervention to minimize the risk of human infections associated with the consumption of raw fruit and vegetables contaminated with foodborne pathogens is the application of chemical treatments. The increased resistance of attached microorganisms to sanitizing agents may partially explain the observed limited effect of disinfection treatments. A clearer understanding of the process of bacterial adhesion to fruit surfaces is essential to devising more effective methods for removal. The objective of this research was to evaluate the effects of inoculum size, relative humidity, ripening stage, and storage temperature on the attachment of *S* Montevideo to the surface of tomatoes and tomatillos.

The influence of inoculum populations and environmental factors on attachment of *Salmonella* Montevideo to the surface of tomatoes and tomatillos was evaluated. To study the effect of inoculum size, red, ripe tomatoes were spot inoculated with bacterial suspensions (10^5 and 10^8 cfu/fruit) and stored at 22°C under 100% relative humidity. The effects of temperature (12, 22, and 30°C) and relative humidity (75, 85, and 97%) on attachment of the pathogen (10^7 cfu/fruit) on tomatoes (red and green) and ripe tomatillos were also evaluated. Inoculated fruit were stored for 90 min at all combinations of temperature and relative humidity, and after washing with water the number of cells attached to the surface was determined. *S*. Montevideo attached to the surface of tomatoes within 90 min. A direct correlation between the number of attached cells and the population in the inoculum was observed. The percentage of cells that attached immediately after inoculum was approximately 0.3% for the test products. After storage for 90 min at various temperature/relative humidity conditions, the number of adhering cells ranged form 4.0 to 5.4 $\log_{10}$ cfu/fruit (1.2% of inoculum). Both the type of product and the temperature/relative humidity condition had a significant ($P \leq 0.05$) effect on attachment of *S* Montevideo to the surfaces of tomatoes and tomatillos. Scanning electron micrographs of the cuticles of inoculated washed tomatoes and tomatillos revealed typical skin cell patterns, and only a few randomly dispersed *S* Montevideo were observed. Deposition *S.* Montevideo on the surface of tomatoes and tomatillos may result in attachment and subsequent colonization under suitable conditions.

FACTORS AFFECTING SURVIVAL, GROWTH, AND RETRIEVAL OF *SALMONELLA* POONA ON INTACT AND WOUNDED CANTALOUPE RIND AND IN STEM SCAR TISSUE

(L. R. Beuchat and A. J. Scouten)

The first documented outbreak of salmonellosis in the U.S. that was associated with consuming cantaloupes was reported in 1990. Consumption of cantaloupes imported from Mexico and Central America was linked to 295 cases of *Salmonella enterica* serotype Chester infections in 30 states. In the summer of 1991, more than 400 cases of *Salmonella enterica* serotype Poona infections in the U.S. and Canada were associated with the consumption of cantaloupe. Twenty-five cases of *Salmonella enterica* serotype Saphra infections in the U.S. were linked to cantaloupes imported from Mexico in 1997 and 22 cases of *Salmonella enterica* serotype Oranienburg infections in Canada in 1998 were attributed to consuming cantaloupes. Subsequent outbreaks of *S.* Poona infections in the U.S. and Canada have been associated with eating cantaloupes grown in Mexico. A survey to determine the presence of foodborne pathogens on fresh produce imported to the U.S. revealed that 11 of 151 (7.3%) cantaloupes were contaminated with *Salmonella* or *Shigella*. A survey of domestically grown fruits and vegetables reported the presence of *Salmonella* on 3 of 92 (3.3%) cantaloupes. Clearly, the pathogen can be found on the surface of cantaloupes at various points postharvest and is somehow transferred to the edible portion before consumption, resulting in human infections. As few as 150 *Salmonella*/cm^2 of netted rind surface of cantaloupes have been shown to contaminate the edible portion upon cutting.

Several researchers have evaluated sanitizers for their efficacy in killing or removing foodborne pathogens and spoilage microorganisms on the surface of cantaloupe rind and the interior flesh. It is difficult to compare the effectiveness of various sanitizers across, and sometimes within, laboratories because of variations in methodology. Methods used to analyze cantaloupes not intentionally inoculated with *Salmonella* also vary among research and testing laboratories. While the development of a standard method to analyze raw fruits and vegetables for *Salmonella* and other pathogens has been promoted, still there are many factors that could influence the efficiency of retrieval of *Salmonella* from cantaloupes that need to be investigated before an optimum method can be

recommended. We conducted a study to determine the survival and recovery of *Salmonella enterica* serotype Poona from cantaloupe rind as affected by environmental conditions between the time of contamination and analysis. Detection and enumeration of the pathogen as influenced by analytical methods were also investigated. Combinations of preenrichment broth (lactose broth or universal preenrichment broth), enrichment broth (Rappaport-Vassiliadis broth or tetrathionate broth), and selective agar medium (bismuth sulfite agar or xyline lysine desoxycholate agar) for detecting *S*. Poona on inoculated cantaloupes stored at 4°C for 7 days or 21°C for 3 days were equivalent in performance. The use of nalidixic acid as a marker in *S*. Poona and in media used to enhance detection or enumeration of the pathogen by inhibiting background microflora in sanitizer efficacy studies, for example, would not adversely affect its survival on or recovery from cantaloupes. Overall, the composition of the carrier (water or 5% horse serum, a high organic matrix) used to prepare inocula did not influence the number of *S*. Poona recovered from the intact rind surface, wounds in the surface, or the stem scar tissue. Regardless of inoculation site or composition of the carrier, populations on spot inoculated melons stored at 4°C remained constant between 2 and 24 h after inoculation. The pathogen grew within 24 h in wounds of spot- and dip-inoculated cantaloupes stored at 21 and 37°C. The addition of up to 1.0% Tween 80 to 0.1% peptone used to remove *S*. Poona from the rind surface did not adversely affect viability and may have enhanced detachment. Consideration of these observations is recommended when developing a method to test the efficacy of sanitizers in killing salmonellae on the rind surface of inoculated cantaloupes and to detect or enumerate salmonellae that may be natural contaminants.