General Interest

Standardization of a Method To Determine the Efficacy of Sanitizers in Inactivating Human Pathogenic Microorganisms on Raw Fruits and Vegetables

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ABSTRACT

The efficacy of sanitizers in killing human pathogenic microorganisms on a wide range of whole and fresh-cut fruits and vegetables has been studied extensively. Numerous challenge studies to determine the effects of storage conditions on survival and growth of pathogens on raw produce have also been reported. Results of these studies are often difficult to assess because of the lack of sufficient reporting of methods or, comparatively, because of variations in procedures for preparing and applying inocula to produce, conditions for treatment and storage, and procedures for enumerating pathogens. There is a need for a standard method to accurately determine the presence and populations of pathogenic microorganisms on produce. The adoption of standard, well-characterized reference strains would benefit a comparative assessment of a basic method among laboratories. A single protocol will not be suitable for all fruits and vegetables. Modifications of a basic method will be necessary to achieve maximum recovery of pathogens on various types of produce subjected to different sanitizer or storage treatments. This article discusses parameters that must be considered in the course of developing a basic standard method against which these modifications could be made.

Documented outbreaks of illness associated with raw fruits and vegetables and unpasteurized fruit juices have occurred with increased frequency in recent years (8, 9). Contamination of raw produce with pathogenic and non-pathogenic microorganisms can occur at any of several points from the field through the time of consumption. Given sufficient time at an appropriate temperature, some pathogens can grow to populations exceeding $10^7$ CFU/g of produce. Many researchers have investigated conditions that affect survival and growth of pathogens on raw produce. Others have evaluated the effectiveness of a wide range of chemical disinfectants in killing pathogens on raw fruits and vegetables (2). However, it is difficult to compare the results of these studies because substantial variations exist in inoculum growth conditions; preparation of inoculum; methods for inoculation; treatment, processing, or storage; and procedures used to detect or enumerate pathogens on raw produce.

There is an urgent need for the development and validation of standard methods to accurately determine the presence and numbers of a wide range of pathogenic microorganisms on raw fruits and vegetables. These methods can then be applied to determine survival and growth characteristics of pathogens in challenge studies and the efficacy of antimicrobial treatments in killing pathogens the produce may harbor. The overall objective would be to develop and validate, using an interlaboratory collaborative approach, a basic experimental protocol that could be modified according to various groups of fruits and vegetables, but used in a standard way to test for the presence and populations of pathogenic microorganisms and for the effectiveness of antimicrobials in killing these microorganisms. Several factors should be evaluated and numerous questions are posed when developing a standard method (Table 1). These factors include the type of produce and pathogen to be examined, population of pathogen in the inoculum, composition of the carrier for the inoculum, and conditions for storing produce between the time of application of inoculum and treatment with disinfectant. The time and temperature of exposure of produce to chemical treatment, procedures for washing produce after application of disinfectant, and procedures for removing and enumerating viable cells of pathogens after treatment should be standardized.

A single detailed experimental protocol will not likely be suitable for all fruits and vegetables. Rather, modifications of a basic protocol for groups of fruits and vegetables

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TABLE 1. Considerations when developing a standard method(s) for determining the efficacy of sanitizers in killing pathogens and survival and growth of pathogens on fruits and vegetables

| Type of produce | Whole or cut  
| Washed, brushed, waxed, or oiled  
| Botanical part (fruit, leaf, stem, flower, root, tuber)  
| Pathogen of interest | Gram-negative or gram-positive bacteria, parasite, or virus; mixture of strains or a single strain  
| Marker or no marker  
| Conditions for preparing inoculum  
| Number of cells in inoculum  
| Procedure for inoculation | Composition of carrier  
| Temperature of produce and inoculum  
| Dip, spray, or spot inoculum  
| Temperature and relative humidity between time of inoculation, testing, and analysis  
| Procedure for evaluating test condition | Define treatment, condition, or sanitizer  
| Method for measurement of concentration and activity  
| Temperature of produce and treatment condition or sanitizer  
| Dipping, spraying, fogging, or atmospheric  
| Agitated, rubbed, or static condition during exposure  
| Time of exposure of inoculated produce to sanitizer or condition  
| Ratio of sanitizer to produce sample  
| Retrieval of pathogens | Sample weight, size, or number of pieces  
| Composition of diluent  
| Blending, homogenizing, macerating, or washing  
| Time of treatment  
| Composition of neutralizer (for sanitizer studies)  
| Detection and enumeration media  
| Conditions for incubating plates and broth  
| Confirmation procedures  
| Reporting results | Number of replicates and samples per replicate  
| CFU/g, CFU/cm², CFU/piece, fraction negative  
| Appropriate statistical analysis and interpretation

will be necessary to enable the most accurate detection or enumeration and to accurately determine the efficacy of disinfectants. Protocol modifications will be necessary for a yet-to-be-determined number of fruits and vegetables defined according to differences in surface morphology and hydrophobicity, internal tissue composition, and conditions of processing, e.g., washing, brushing, or waxing, to which produce had been previously subjected. Presented herein are observations on method development and suggestions concerning future directions that might be taken on an international scale to establish standard methods to accurately detect or enumerate pathogens on raw fruits and vegetables.

ORGANISM AND PREPARATION OF INOCULUM

Preceding evaluation of the efficacy of sanitizers or appropriateness of experimental protocols for challenge studies to determine the survival or growth of pathogens is the development and validation of a standard method for detection and enumeration of pathogens on raw produce. Optimum conditions for culturing pathogens that may be present on or in fruits and vegetables differ, thus requiring different methods for preparing cells to be used as inocula for these studies. Overall, however, several types of pathogenic bacteria that may be present on produce can, in some respects, be considered similar with regard to developing a standard protocol to determine their presence and numbers, survival, growth, and optimum method for detection or enumeration. Details describing conditions for culturing each microorganism will not be covered herein. Rather, broad considerations that need to be addressed in developing a standard protocol will be discussed. Attention to factors that must be considered in developing a standard method for detecting and enumerating pathogenic bacteria will be stressed.

The selection of the strain or strains of a particular pathogen to be used in studies designed to determine the number of cells present or the efficacy of a decontamination treatment is extremely important. The use of standard, well-characterized reference strains would benefit comparative assessment of a basic method among laboratories. A minimum of five strains, preferably recently isolated from produce or other plant materials and from patients with illness associated with consumption of a raw fruit or vegetable, at approximately equal populations in a mixed inoculum is recommended. If there are differences in the ability of one or more of these strains to survive or grow on produce held under various environmental conditions or if there are differences in susceptibility to decontamination treatments, the most robust strain(s) will prevail. If only one strain is used as an inoculum, that strain should have been evaluated against several other strains for resistance to the test conditions. Otherwise, the use of a single strain that may be less tolerant to test conditions would result in an inaccurate picture of the behavior of the pathogen. Strains used to prepare mixed-strain suspensions should be examined for potential reactions against each other that may be caused by inhibitors, e.g., lytic agents.

Each pathogen should be cultured in a standard broth or agar medium at a specific temperature for a specific time. The temperature at which bacterial pathogens are grown for preparing inocula should be representative of the temperature at which they had grown before contaminating produce, e.g., at 37°C if bovine feces is the vehicle, or at the temperature at which they will be exposed during storage, e.g., 5 to 12°C for fresh-cut produce or 20 to 25°C for some types of whole fruits and vegetables. Several transfers of cultures should be made before the day of inoculum preparation. Although this practice may result in strains with reduced environmental stress tolerance, because of adaptation to rich nutrient media with repeated broth culturing, it is most desirable to prepare inoculum of uniform cell type. The specific nature of the study being conducted should be carefully evaluated to determine if potential genetic selection will affect the predictive value of the results for environmentally acquired pathogens. Because stationary phase bacterial cells are generally more tolerant to environ-
mental stresses than are logarithmic growth phase cells (6), the former should be used for studies to develop optimum procedures for maximum recovery from inoculated produce. Bacterial strain markers may be desirable to facilitate the recovery of cells in enrichment broth or counting colonies on selective or nonselective direct plating media, which otherwise may support large numbers of background microflora. For example, adaptation of Gram-negative pathogens to nalidixic acid (50 μg/ml) has been successfully used in achieving these objectives. Other markers include rifampicin (80 μg/ml), particularly for isolating pathogens from inoculated fruits and vegetables that have significant adhering soil, and plasmid-borne or chromosomally stabilized markers, such as expressed fluorescent proteins with various chromophoric properties. Whichever marker is selected, it is critical to assess the impact of marker selection or introduction on growth rate, stress tolerance, and recovery efficiency on enumeration media. It is also necessary to characterize the stability of the marker for at least 10 generations, without selection, for studies where multiplication on inoculated produce is being evaluated or for recovery methods that include a nonselective growth enrichment step.

Methods for determining survival or inactivation of viruses and parasites on produce pose unique problems. Although many researchers have investigated the survival and inactivation of enteric viruses, many obstacles still remain in standardizing methods to determine the efficacy of sanitizers. First, many of the freshly isolated foodborne viruses, such as hepatitis A and Norwalk viruses, are not culturable, and thus cannot be propagated in sufficient quantities for appropriate investigation, nor can they be quantitated by the plaque assay. Only a few strains belonging to certain virus groups, e.g., poliovirus, have been laboratory adapted to grow in tissue culture and be quantitated by the plaque assay. Even though some of these viruses belong to the same family, they can vary widely in their resistance to various chemical and physical stresses. These adapted strains can, at best, be representative of only their parental wild type, rather than other members in the same family. The modest success in propagating some of the foodborne viruses has limited studies to only a few well-adapted strains, which may or may not be similar in behavior to other isolates of the same virus. The application of novel and rapid molecular methods to detect viruses in foods, although quite sensitive, cannot be used to quantitate virus titer or to differentiate between infectious and noninfectious virus particles (7), thus posing unique challenges in method development and standardization.

Studies to determine the survival of parasites on raw produce as affected by sanitizers or various environmental conditions during storage are lacking. A major constraint to these studies is the limited supply of oocysts (5). Currently, infected humans are the only source of Cyclospora cayetanensis oocysts, making research to determine susceptibility to stress conditions limited to only a few laboratories. The lack of sensitive laboratory methods for quantitating and assessing the viability of oocysts also hampers studies focused on determining the efficacy of inactivation treatments and the influence of processing, packaging, and storage conditions on survival of oocysts.

Potential vehicles of pathogens for contaminating fruits and vegetables include dust, rain water, irrigation water, sewage, soil, feces, decayed plant material, contact surfaces, workers at any point from harvesting to preparation in a food service setting, and consumers (1). Whatever the vehicle, cells of pathogens are likely to be entrapped in organic material. For this reason, to simulate practical conditions, the carrier for the inoculum should contain organic material. A 5% solution of horse serum albumen can be used as a carrier in studies to determine the efficacy of sanitizers, although an aqueous peptone solution may also be appropriate. It is important to not use a buffer or other carriers containing salts or other chemicals that could be detrimental to cells after the inoculum has dried on the surface of test produce. Cells in broth cultures should be washed in peptone water and pellets should be resuspended in the organic carrier shortly before using as an inoculum. For challenge studies designed to determine the survival or growth of pathogens on produce, the carrier may be a source of nutrients, thus complicating interpretation of results. The use of two carriers, one with and one without organic material, for test cells may be useful in generating information to discern the effects of nutrients on survival and growth of test pathogens.

The desired number of CFU of the bacterium per ml of inoculum is dictated by the objective of the study. Two or three levels of inocula, ranging from $10^0$ to $10^7$ CFU/g or CFU/cm$^2$, may be applied to facilitate determination of the efficiency of retrieval techniques or efficacy of disinfectants. Decontamination studies require the use of high numbers of cells in the inoculum to enable measurement of several $\log_{10}$ reductions in CFU/g or CFU/cm$^2$. Challenge studies require inocula containing low numbers of cells to enable measurement of growth during subsequent storage under conditions simulating commercial practice.

**TYPE OF FRUIT OR VEGETABLE**

A single procedure to efficiently remove microbial cells from all types of fruits and vegetables for the purpose of detection or enumeration would be ideal. However, for several reasons, this is not an achievable goal. Great differences in size, shape, and surface morphologic features of fruits and vegetables complicate the sampling protocol. The ratio of surface area to weight varies substantially, raising a question of whether to report data on the basis of CFU/g or CFU/cm$^2$. The procedure for preparing the sample also must be addressed. Homogenization of a standard weight, say 25 g, of a fruit or vegetable using a standard volume of diluent would be a simple approach to sample selection and preparation. Problems associated with homogenized, blended, or macerated plant tissues, however, include the potential lethal effect of naturally occurring antimicrobial compounds and phytoalexins against pathogens or other microflora being enumerated. When microbial cells on the surface of produce come in contact with organic acids or other antimicrobials naturally present in plant tissue fluid, or produced on rupture of cells or invasion with
insects or molds, death may occur (11). Numerous studies have described inhibitory or lethal activities of naturally occurring antimicrobials against foodborne microorganisms, including species capable of causing human illness. Thus, although blending, homogenizing, or macerating may be acceptable in preparing samples of some types of fruits and vegetables, a simple surface washing without rupturing of plant cells may be required for other types of produce.

Categorizing fruits and vegetables according to their amenability to homogenization versus surface washing to retrieve microbial cells, based on considerations of antimicrobials in plant tissues, would be the first step in developing a sample preparation protocol. The variable presence of inhibitory residues from crop management practices specific to the source of fruits or vegetables may be a consideration. Within the group of whole fruits and vegetables that require surface washing, microorganisms may be most effectively retrieved by rubbing the surface of the fruit or vegetable. Examples would be tomatoes, pepper, avocados, watermelons, oranges, and other produce with a fairly rigid surface. Sonication of samples is an alternative method for removal of surface microorganisms with minimal tissue disruption. For leafy and floret vegetables, as well as strawberries, drupes, and other produce with surface tissues easily ruptured by rubbing, agitation (mechanical shaker or manually shaking) or sonication in a wash fluid with standard composition for a set period may be necessary. In an attempt to avoid too many modifications of a standard sample preparation protocol, the sample weight or number of pieces should be standardized. Calculations of CFU/g could be converted to CFU/cm² using a conversion table listing estimated values for specific fruits and vegetables categorized as spheres, cylinders, or two-sided planes. In some cases, it may be appropriate to calculate microbial populations per piece of fruit or vegetable.

Retrieval of microbial cells from fruits and vegetables that have been mechanically cleaned by brushing or that have been waxed or oiled may be more difficult compared with retrieval from untreated produce. Cells entrapped in bruised tissue, waxes, and oils may not be removed or homogeneously dispersed in homogenates or wash fluids, thus resulting in an underestimation of populations. Modifications in the sample preparation protocol may be necessary to maximize release of cells from all tissues and the naturally occurring waxy cutin layer and waxes or oils that may be applied to enhance appearance or extend the shelf life of certain fruits and vegetables.

PROCEDURE FOR INOCULATION

Procedures for inoculating fruits and vegetables with pathogens include dipping or spraying with a suspension of cells or applying a known volume of cell suspension. If the suspected point of contamination in a commercial situation is an immersion process, then dipping the produce in the test cell suspension may be the most appropriate method for inoculation. The major problem associated with inoculation by dipping or spraying is that the number of cells actually applied or adhering to the produce is not known. The acquired inoculum among individual test units is often highly variable. Using dip or spray methods requires the analysis of a large number of units for each treatment, since random error values are unpredictably large. Thus, efficiency of recovery or log₁₀ changes in viable populations during subsequent storage or as a result of treatment with a disinfectant cannot be accurately calculated. Also, infiltration of the inoculum into exceptionally porous areas on the produce surface, e.g., stem core tissue or cut tissue (3, 10), can result in conditions that may promote or inhibit growth or protect against contact with decontaminants, particularly those with little or no surfactant activity. Alternatively, fruits and vegetables can be inoculated by applying a known volume, e.g., 10 or 50 µl, of inoculum of known cell density to the surface. This type of inoculation would be representative of contamination from a point source, e.g., from contact with soil, workers’ hands, or equipment surfaces.

A negative differential between the temperature of the produce and the temperature of the inoculum, i.e., the temperature of the produce is higher than the temperature of the inoculum, can result in enhanced infiltration of microorganisms into the tissue (4, 12). It is important to select a standard temperature at which both the produce and the inoculum are adjusted at the time of inoculation. Otherwise, survival, growth, sensitivity to disinfectants, and efficiency of retrieval of cells may be affected. At least three replicate experiments, each including two or more samples for each set of test parameters should be done. In some instances, more samples may need to be analyzed, depending on specific objectives.

In studies to determine the efficiency of retrieval of cells or efficacy of disinfectants, the inoculum applied to produce should be dried for a standard period at a standard temperature and relative humidity before retrieval is attempted or treatment is administered. Fluctuations in temperature and relative humidity should be minimized between the time of drying and treatment with disinfectant. Negative controls should always be included.

EFFICIENCY OF RETRIEVAL AND EFFICACY OF DECONTAMINATION TREATMENT

The development of a standard protocol for detecting or enumerating a specific pathogen on produce should include experiments to validate recovery based on a known number of cells applied. Using a specific volume of inoculum containing a known number of test cells, this can be achieved. Although some cells may die during the drying period following application of inoculum, spot inoculation is superior to dip or spray inoculation, which do not enable measurement of the number of cells adhering to the produce.

The efficiency of retrieval of microbial cells naturally occurring on produce is not easily determined, simply because the actual number of viable retrievable cells is not known. One is forced to compare various combinations of sample weights, wash fluids, diluents, homogenization or washing treatments, and neutralizers (in the case of chemical sanitizer tests), then choose the set of test parameters that yields the highest number of microorganisms recov-
ered. This can be an exhausting task. Nevertheless, some protocols have been demonstrated to be more efficient than others, and a single basic protocol should be selected for use in all laboratories.

Establishing a protocol for efficient recovery of pathogens or groups of microorganisms from fruits and vegetables is paramount before proceeding to experiments designed to determine the efficacy of treatment with disinfectants. Once a basic retrieval protocol is in place, a standard protocol for chemical and physical decontamination treatments can then be developed. The protocol for chemical decontamination should include standard ratios of weight to volume (produce to treatment solution or atmosphere, whether applied as a dip, spray, or fog), treatment solution concentrations, treatment time and temperature, and type and volume of neutralizer to be used at the end of the treatment time. Whether the produce should be static, agitated, or hand rubbed during chemical treatment should be defined. If agitated, a procedure, e.g., placing the produce and treatment solution on a mechanical shaker or manually shaking, should be standardized. Procedures for separating the produce from the chemical treatment solution, washing with a specific neutralizer, and subsequent homogenization or washing in a specific volume of a given diluent should likewise be standardized. In the case of physical decontamination treatments, standardization of conditions used to apply the potentially lethal force, i.e., temperature, irradiation, or pressure, would also facilitate comparison of observations across laboratories.

A neutralization step is not necessary in a standard protocol to measure the efficacy of physical treatments, but, like the protocol for determining the efficacy of chemical disinfectant treatments, standardization of diluent composition, ratio of produce weight to diluent volume, homogenization or washing procedure, enrichment and direct plating media, and incubation conditions will be necessary.

**PROCEDURE FOR DETECTION AND ENUMERATION**

Although the basic protocol for detecting or enumerating pathogenic bacteria on fruits and vegetables should be standardized, the selection of specific direct plating and enrichment media, as well as conditions for incubation and procedures for confirmation of isolates, will obviously differ, depending on the pathogen. It is not our intention to recommend specific media, incubation conditions, or confirmation techniques for each pathogen that may be present on raw produce but rather to stress the necessity of selecting a single procedure for use across laboratories.

The optimum protocol for retrieving pathogens from fruits and vegetables may differ, depending on whether analysis of the surface, tissue, or a composite of both is desirable. Washing, blending, homogenizing, stomaching, macerating, or grinding are among the choices to process samples for direct plating or enrichment. The entire piece or only a portion of the fruit or vegetable may be selected for analysis, but the procedure needs to be standardized in terms of weight examined or excision technique. Likewise, the composition, pH, and ratio of diluent to sample need to be consistent at least within each fruit and vegetable group. A standard time and temperature for preparing samples for plating or enrichment should be used.

The presence of stressed or injured microbial cells on fruits and vegetables should be recognized, and resuscitation techniques may be necessary. Protection of cells on the surface of produce that, for example, may be debilitated by desiccation or as a result of exposure to a harsh acidic environment is important if these injured cells are to be detected. Adjustment of the pH of homogenates may be necessary to protect pathogens against potentially lethal acidic environments.

**REPORTING RESULTS**

Given the variability in parameters naturally intrinsic to fruits and vegetables, as well as variation in types and numbers of microorganisms and amount of soil and organic matter present on produce surfaces, the procedure for selecting samples for sanitizer efficacy or challenge studies is important. It will be necessary to include a sufficient number of replicates with sufficient numbers of whole fruits and vegetables or cut produce samples to enable appropriate types of statistical analysis to be applied to the data generated. The experimental design should enable a level of statistical analysis rigorous enough to deal with the complexities associated with microbiological testing. Traditional methods of bacteriological or mycological analysis of foods report results in terms of CFU/g or CFU/cm². Processing technologies may be designed to achieve a certain log₁₀ reduction in number of a specific pathogen or several pathogens based on weight or volume of the product. This approach is not reasonable for all fruits and vegetables because of the tremendous variation in weight-surface area (g/cm²) among various types of produce. Figure 1 shows the relationships between the weight (g) and surface area (cm²) of iceberg lettuce and tomato. Although the ratio of weight to surface area will vary, depending on the thickness of the lettuce leaf and variations in shape of both vegetables, this figure simply illustrates that large differences in
ratios can exist among fruits and vegetables. Relationships of weight and surface area of other fruits and vegetables with geometric configurations other than a two-sided plane (lettuce) and sphere (tomato) would fall somewhere between these extremes. A decontamination process designed to achieve, for example, a 3-log$_{10}$ reduction in CFU/g of lettuce or tomato would result, respectively, in approximately 0.114- and 18-log$_{10}$ reductions in CFU/cm$^2$. A 3-log$_{10}$ reduction in CFU/cm$^2$ of lettuce or tomato would result, respectively, in approximately 78.9- and 0.5-log$_{10}$ reductions in CFU/g.

A decision needs to be made concerning a standard procedure for calculating and reporting populations of microorganisms on fruits and vegetables. Guidelines or limits for maximum numbers of pathogens on untreated produce should reflect this standard basis for calculation. Likewise, the number of log$_{10}$ reductions in CFU resulting from a decontamination process should be based on a standard procedure for calculation. Whichever procedure is used, differences in guidelines or limits and log$_{10}$ reductions achievable by disinfectant treatment for specific pathogens on various groups of fruits and vegetables, according to their geometric characteristics, will be necessary. Finally, data need to be subjected to appropriate statistical analysis to determine significant differences in populations of test pathogens recovered from produce subjected to various treatment or storage conditions.

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