



Occurrence of Bacteria in Dishcloths Used in Restaurants and Survival of Respiratory Viruses on Produce

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OCCURRENCE OF BACTERIA IN DISHCLOTHS USED IN RESTAURANTS
AND SURVIVAL OF RESPIRATORY VIRUSES ON PRODUCE

By

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RESTAURANTS AND SURVIVAL OF RESPIRATORY VIRUSES ON PRODUCE

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DEDICATION

This dissertation is dedicated to God, who gave me life and the necessary tools to achieve one more goal in my life. ! To him all the Honor and Glory! Thank you Lord!

To my parents Leonardo and Kitty (†); mom, wherever you are, I know you have been always praying for me and my family. I thank you both, because you set the example at home of staying united, working hard, loving God, and keep studying and reaching goals, no matter the age.

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ABSTRACT

The first study was designed to determine the occurrence of bacteria in dishcloths used in restaurants and bars. Coliforms were isolated from 89% of dishcloths and 70% of tabletops. *Escherichia coli* was isolated from 54% of dishcloths and 20% of tabletops. The numbers of heterotrophic bacteria (HPC) and coliforms were higher in bars than in restaurants. The levels of HPC found in dishcloths were 25-fold and coliforms were 60-to 120-fold lower than the levels found in dishcloths in previous home studies. The most commonly isolated genera from dishcloths in restaurants and bars differed from those in homes. The numbers of HPC on restaurant tabletops were 45-fold greater after cleaning than prior to cleaning. The mandatory use of sanitizers in restaurants and bars may therefore have reduced contamination levels and caused a shift in the microbial populations present in food service establishments.

The second study was designed to determine the recovery efficiency and the survival of two respiratory viruses on produce and was compared to the survival of the enteric poliovirus 1. Adenovirus was recovered with an efficiency of 56%, 32% and 35% from lettuce, strawberries and raspberries, respectively. Coronavirus was recovered from lettuce with an efficiency of 19.6%, but could not be recovered from strawberries. Poliovirus was recovered from lettuce with an efficiency of 76.6% and 0.06% from strawberries. The survival of the viruses was observed for up to eight days. Adenovirus survived the longest on raspberries, with a \log_{10} reduction of 0.61, followed by 1.68- and 1.75- \log_{10} reductions on strawberries and lettuce, respectively. Coronavirus declined by

0.41- \log_{10} after two days and $>1.34 \log_{10}$ by day 4 on lettuce. The enteric poliovirus 1 survived longer on produce, decreasing by only 0.37- \log_{10} on lettuce and 1.30- \log_{10} on strawberries.

A microbial risk assessment was performed to assess the risk of infection from ingesting 1, 10, and 100 particles of adenovirus on lettuce. The estimated risk of infection by ingesting these numbers were 1:2000, 1:200, and 1:20, respectively and increased in a proportional way as the number of servings was increased from one to ten and 365 servings of lettuce.

CHAPTER 1

OCCURRENCE OF BACTERIA IN DISHCLOTHS USED IN RESTAURANTS

The manuscript entitled, “Identity and Numbers of Bacteria Present in Tabletops and in Cloths Used to Wipe down Tabletops in Public Restaurants and Bars” was published in *Food Protection Trends* (2006) 26(11):786-792. However, in order to provide coherency, it was included in the body of the dissertation as a manuscript. The permission from the copyright holder are appended in Appendix A. This work was done in collaboration with the Clorox Company, who provided some of the samples collected in sites outside the Tucson, AZ area. Dr Kelly Bright assisted in data analysis and in the preparation of the manuscript. The dissertation author carried out all the microbiologic analysis and most of the sampling done in Tucson and Nogales AZ. The dissertation author is responsible for all the research presented in the manuscript.

1.1 Literature Review.

Foodborne disease consumes a substantial amount of health care resources and causes considerable mortality and morbidity throughout the world (Panisello et al., 2000). Unsafe foods cause an estimated 76 million illnesses, 325,000 hospitalizations and 5,020 deaths annually in the United States (Mead et al., 1999). It has been estimated that viruses are responsible for 67% of all foodborne illness, bacteria for 30%, and parasites for 3%. Pathogens mostly associated with foodborne outbreaks are norovirus, *Campylobacter* spp., *Escherichia coli*, *Salmonella* non-typhoidal, *Staphylococcus aureus* poisoning, *Toxoplasma gondii*, and *Listeria monocytogenes* (Mead et al., 1999). According to the

UK Health Protection Agency, an estimated of 1.3 million people in England and Wales suffer from the effects of food poisoning each year with an estimated 500 deaths (IFH, 2007).

The Center for Science in the Public Interest (CSPI) in the United States, maintains a database of foodborne illness outbreaks that have been linked to specific foods. CSPI identified a total of 5,778 outbreaks of illness linked to specific foods, involving 168,898 individual illnesses that occurred between 1990 and 2006 (DeWaal, 2008). Table 1.1.1 shows the number of outbreaks of illness linked to specific food. It is important to note the higher number of cases of illness caused by produce, in comparison with the total number of outbreaks. Bacterial pathogens cause more than three-fourths of outbreaks linked to beef, dairy, eggs, luncheon and other meats, pork, and poultry. Outbreaks with multi-ingredient foods are more commonly caused by bacteria (58%), but are also often caused by viruses (40%).

Table 1.1.1 Outbreaks of illness linked to specific foods
in United States between 1990 and 2006.

Foods	Outbreaks	Illness
Seafood	1,140	11,809
Produce	768	35,060
Poultry	620	18,906
Beef	518	14,191
Eggs	351	11,143

(De Waal, 2008)

The most prominent bacterial pathogens identified in the outbreak data were *Salmonella* (23% of all outbreaks), *Clostridium* (11%), and *Staphylococcus* (8%). Norovirus, which typically causes non-fatal illness, causes 90% of all virus outbreaks identified, accounting for 22% of outbreaks in the entire database. Both *E. coli* and *Bacillus* cause 5%, and *Campylobacter* causes 3% of all outbreaks, respectively (DeWaal, 2008).

Five-hundred and thirty general foodborne outbreaks of food poisoning were reported in England and Wales between 1992 and 1996. Improper cooking, inadequate storage, cross-contamination and use of raw ingredients in the preparation of food were the most common factors contributing to outbreaks (Panisello et al., 2000). From the 530 outbreaks, inadequate handling contributed with 166 (31.3%), and included food handler 48 (9.1%) and cross-contamination 118 (22.3%). *Salmonella* spp. was identified in 18.7% of all the cases in which cross-contamination was the contributing factor, and poultry and red meat were among the most important vehicles of transmission reported (Panisello et al., 2000).

To assess the degree of bacterial transfer from raw meat to the salad, chicken breast fillets were inoculated with a known contamination level of *Campylobacter jejuni* and *Listeria casei* and salad contamination levels determined by De Jong et al. (2008). Salads were prepared according to different cross-contamination scenarios and different washing procedures. High contamination levels of both microorganisms were observed in salads when cross-contamination via cutting board, cutlery, or hands was not prevented. Cross-contamination of *C. jejuni* via cutting board was strongly reduced when rinsing the

cutting board for 10 seconds under hot running water, but using hot water and detergent resulted in higher contamination levels of the salads than only using hot water as a rinse; using a cold water rinse hardly affected cell counts compared with non-washed cutting boards. Cutlery was adequately cleaned in most cases using a cold water rinse considering *C. jejuni*, while this effect was only partly achieved when cutlery was washed using hot water and soap for *L. casei*. Cross-contamination of *C. jejuni* via hands was reduced when using cold water and soap during hand washing. *L. casei* was only poorly removed by rinsing with only cold water but the use of soap resulted in a mean reduction of another 2-log₁₀ cfu/salad, but high numbers were still occasionally recovered (De Jong et al., 2008).

To assess the occurrence of cross contamination, the preparation of salad in a household kitchen was simulated; starting with washing *C. jejuni* contaminated vegetables in tap water, then cutting the vegetables on a cutting board, followed by slicing cucumber and blanching (heating in hot water) the vegetables in 85°C water (Chai et al., 2008). It was found that 30.1 to 38.2% of *C. jejuni* transfer from vegetables to wash water, 26.3 to 47.2% from wash water to cucumber, 1.6 to 10.3% from vegetables to cutting board, and 22.6 to 73.3% from cutting board to cucumber, suggesting that wash water and plastic cutting boards are potential risk factors in *C. jejuni* transmission (Chai et al., 2008).

Although it is recognized that the main factors determining food safety at home are adequate cooking, proper storage, personal hygiene and preventing cross-contamination, there is still up to 87% of reported foodborne outbreaks associated with

food prepared or consumed in the home (Redmond and Griffith, 2003; van Asselt et al., 2008). For a risk of foodborne infection to occur, two criteria must be met: the bacteria present must be pathogenic, and a route needs to exist by which the bacteria can move from a surface onto food (Hilton and Austin, 2000). We are often reminded that it is important to separate raw and cooked foods in the kitchen, and that hands are the most important “superhighway” for spreading germs, but we tend to forget that cleaning cloths and other cleaning utensils can easily spread germs to our hands and to clean food contact surfaces if we do not look after them properly (IFH, 2007).

Some studies have indicated that cross-contamination from raw products via hands, cleaning cloths or sponges and utensils to foods ready to eat contributed to the occurrence of outbreaks of foodborne salmonellosis and campylobacteriosis in the United States and in other industrialized countries (Brown et al., 1988; Cogan et al., 1999; de Jong et al., 2008; Medeiros et al., 2008).

When good hygiene practices are applied, food particles are usually cleaned from the surface; however, bacteria attached to these surfaces are not visible to the eye and may therefore not be removed (Kusumaningrum et al., 2003). In addition, many surfaces in food processing plants can support bacterial biofilm formation and although stainless steel appears smooth to the naked eye, it is actually quite rough with many distinct flaws that could harbor or provide attachment sites for bacterial cells (Zottola and Sasahara, 1994; Annous et al., 2009).

During the cleaning process of equipment, utensils, sinks, food preparation areas, etc. in the kitchen, the washing steps are done with the use of sponges to eliminate food

residues. As a consequence, part of the food adheres to the sponge surfaces, so residues, together with the moisture retained in the sponges, offer a favorable environment for bacterial growth (Erdoğan and Erbilir, 2005). Dishcloths are often used to wipe down food preparation surfaces and therefore may become contaminated with the bacteria originally present on raw food; later, the cloth may be put to further use without prior disinfection, resulting in further contamination of surfaces. The ability of the dishcloth to act as a vehicle for this contamination will ultimately depend on whether these organisms can be released back into the kitchen environment (Hilton and Austin, 2000).

Several studies have indicated that various bacteria, including *E. coli*, *S. aureus* and *Salmonella* spp., survive on hands, sponges/cloths, and utensils for hours or days after initial contact with the microorganism (Kusumaningrum et al., 2003), and that wet cloths and cleaning utensils together with hands and food contact surfaces, are important elements in cross-contamination (Scott and Bloomfield, 1990b; Enriquez et al., 1997a; Chaidez and Gerba, 2000).

Josephson et al. (1997) conducted a three-part study in which the primary objective of the first phase was to establish the incidence and concentrations of specific pathogens such as *Salmonella* and *Campylobacter* present in 10 “normal” kitchens not currently using antimicrobial kitchen cleaners. Eight locations within the kitchens were monitored for: total heterotrophs, Staphylococci, *Pseudomonas*, total coliforms and fecal coliforms. Almost all locations at all households exhibited contamination, with the sink and sponge samples exhibiting large bacterial numbers. The fecal coliform concentrations in sink and sponge samples were very high, with 63 and 67% of all samples positive,

respectively. *E. coli* was detected in 16.7% of all sink surfaces and 33.3% of all sponges. *Salmonella* was detected once and *Campylobacter* on two occasions (Josephson et al., 1997).

In order to determine the extent to which survival of organisms on cloths and laminate surfaces may be associated with cross-contamination via the hands, cloths and stainless steel surfaces, Scott and Bloomfield (1990b) inoculated both, clean and soiled laminate surfaces (as plates) and cloths with several bacterial organisms. They also determined the transfer rates from soiled laminated surfaces via the fingertips and from cloths to fingers and laminated surfaces (Scott and Bloomfield, 1990b). They found that a substantial proportion of the inoculum was recovered at 0 and 1 hour from both clean and soiled surfaces while they remained damp. Some species showed an initial increase in numbers between 0 and 1 hour suggesting multiplication on surfaces. Under soiled conditions, *E. coli*, *Klebsiella pneumoniae*, and *Salmonella* spp. survived in significant numbers up to 4 hours, and up to 24 hours for *S. aureus*. For soiled cloths, some species had an initial reduction at 4 hours; however, a substantial regrowth of residual survivors occurred within 24 hours. They also found that significant numbers of *E. coli*, *Salmonella* spp. and *S. aureus* were transferred for 1-2 hours after contamination and up to 24 hours for *S. aureus* via the fingertips. Where contaminated cloths were handled or applied to a clean laminate surface, significant transfer occurred, giving contamination levels “too numerous to count” at 24 and 48 hours.

In another study, Rusin et al. (2002) determined the transfer efficiency of microorganisms from fomites to hands. They found that the water recovered from the

common kitchen sponges contained an average of approximately 3.2×10^8 coliforms/ml in the sponge itself and that after squeezing out a sponge or a dishcloth, the subjects' hands were highly contaminated. The average transfer efficiency of bacteria and phage from dishcloth/sponge to hands was 0.02%. Their results suggest that commonly handled objects that are microbially contaminated can serve as reservoirs of bacteria and viruses that can easily be transferred to the hands through direct contact.

Enriquez et al. (1997b) collected 325 sponges and 75 cotton dishcloths from households in four major cities in the USA to determine the number of total and fecal coliform bacteria and another types of colonizing bacteria. They found average geometric means (GM) of total coliforms for sponges and dishcloths of 1.15×10^5 and 1.31×10^5 cfu/ml, respectively and of fecal coliforms of 4.46×10^2 and 2.03×10^3 cfu/ml, respectively. *Pseudomonas* spp. were the organisms found most frequently in cellulose sponges (35.7%) and dishcloths (31%), with *P. putida* as the predominant species (20.2% in sponges and 24% in dishcloths). Among the Enterobacteriaceae, *Salmonella* spp. were the most commonly found organism in both sponges and dishcloths, followed by *Enterobacter* spp., *Klebsiella* spp., and *Serratia* spp. Presumptive *Staphylococcus aureus* was isolated from 20% of sponges and from 18.6% of dishcloths. Their results demonstrate that cleaning cloths and sponges may be a significant source of pathogenic and opportunistic pathogenic bacteria in the domestic kitchen environment.

In a similar study to determine the occurrence of bacteria in household cleaning tools conducted in a developing country, Chaidez and Gerba (2000) determined the microflora of cleaning tools in household kitchens in Culiacan, Mexico. They collected

and compared 27 commercial cellulose and 23 natural fiber sponges (loofahs, widely used as cleaning tools in domestic kitchens in Mexico), and enumerated total and fecal coliforms, *S. aureus*, and other bacteria present. They found that total coliforms were greater in the cellulose sponges (GM=1.5x10⁵cfu/ml) than in loofahs (GM=1.4x10⁴ cfu/ml). In contrast, for the fecal coliform bacteria, the GMs in cellulose sponges and loofahs were 1x10³ and 1.5x10⁴ cfu/ml, respectively. The organisms most frequently identified were *Aeromonas hydrophila* (19.5%), *Pseudomonas pseudoalcaligenes* (12.2%), and *Burkholderia cepacia* (9.8%), together with *Salmonella* spp. (9.8%), and *K. pneumoniae* (4.9%). Presumptive *S. aureus* was frequently isolated (92%) from both cellulose and natural fiber sponges.

To determine the presence of total and fecal coliforms on kitchen surfaces, in tap water and on the hands of caregivers in households on both sides of the US-Mexico border, Carrasco et al. (2008) collected samples in 135 randomly selected households in Ciudad Juarez, Mexico, and El Paso, Texas. Different surfaces throughout the kitchen and the head of households' hands were sampled. Sponge/dishcloth and drinking water samples were also obtained. Sponge/dishcloth samples were the most commonly contaminated kitchen sites (60%), followed by sink knobs (40%), tables and countertops (28%), and cutting boards (19%). Fecal coliforms were recovered from 14% of the hands of child caregivers, and this indicator was moderately associated with self-reported failure to wash hands after using the toilet.

In a study done by Hilton and Austin (2000) of domestic kitchens in the West Midlands of Birmingham area of England, a combination of "in use" kitchen cloths (54)

and sponges (46) were collected from randomly selected houses and analyzed microbiologically; the ability of the kitchen dishcloth or sponge to transfer bacteria onto a cutting board surface was also determined. Each kitchen cloth or sponge was analyzed for the presence of *Salmonella*, *Campylobacter* and *S. aureus*, and for total viable counts (TVC) of aerobic bacteria. Neither *Campylobacter* nor *Salmonella* was detected on any of the dishcloths collected. *S. aureus* was detected on 4% of sponges, ranging in number from 10^2 to 4.0×10^4 cfu/ml. TVC of the sponges were significantly larger than those of the cloths. This may be as a result of the surface characteristics of the two cloth types. The structure of the sponge, being cavernous in nature, may offer a protective microenvironment where microbial attachment and survival is facilitated compared to the more exposed surface of the cloth which transferred a significantly greater proportion of its bacterial loading than the sponge to the cutting board surfaces. This again may be a result of the microstructure of the sponges which hold trapped microorganisms away from the contact surface, thus preventing transfer. Therefore, this suggests that a contaminated cloth may represent a greater risk of transferring organisms to a food preparation surface than a contaminated sponge.

Foodborne pathogens can persist in a kitchen environment and may be spread using kitchen sponges unless properly disinfected. Disinfection of sponges may be one aspect of kitchen hygiene that decreases the risk of foodborne illness in the home (Sharma et al., 2009). Several studies have been done regarding the effectiveness of detergent washing (Cogan, et al., 2002), chemical disinfection of cleaning cloths and sponges with sodium hypochlorite (Scott and Bloomfield, 1990a; Rusin et al., 1998), with

quaternary ammonium disinfectants (Scott and Bloomfield, 1993), with antibacterial dishwashing liquids (Erdoğan and Erbilir, 2005), the use of self-disinfecting sponges (Enriquez et al., 1997a), and even microwaving and dishwashing sponges (Sharma et al., 2009).

In 1998, The Food and Drug Administration's (FDA) initiated a study to measure the occurrence of food preparation practices and employee behaviors most commonly reported to the Centers for Disease Control and Prevention (CDC) as contributing factors in foodborne illness outbreaks. During inspections of various establishments they observed and documented the occurrence of food from unsafe sources, improper holding/time and temperature, inadequate cooking, poor personal hygiene, and contaminated equipment/ prevention of contamination, they determined the main contributing factors (FDA, 2004).

The 2004 report indicated that in all facility types, the out of compliance percentages remained high for improper holding/time and temperature, poor personal hygiene, and contaminated equipment/prevention of contamination. The improper cleaning and sanitizing of food contact surfaces before use was the item most commonly observed to be out of compliance in each facility type (FDA, 2004). Proper cleaning and sanitization of food contact surfaces is an effective means of preventing cross-contamination. Many different procedures may be involved in the assessment of the management system related to this area. Evaluations should not be restricted to dishwashing procedures but should also include observations on how food preparation tables, cutting boards, and stand-in-place equipment such as slicers and mixers are

cleaned and sanitized between use. The high out of compliance percentage for cleaning and sanitizing food contact surfaces and utensils indicates a weakness in fast food restaurant management systems designed to prevent cross-contamination (FDA, 2004).

The FDA Federal Food Code recommends that linens used in restaurants and retail food stores to wipe down food service areas be soaked in one of the approved sanitizers shown in Table 1.1.2. The purpose of these sanitizer solutions is to reduce at least 5-log_{10} (99.999%) the numbers of representative disease microorganisms of public health importance (FDA-Anonymous, 2005).

Table 1.1.2 FDA approved chemical dishcloth sanitizers for food service establishments.

Sanitizer	Concentration (mg/L)	Time (sec.)	Temperature (°C)	pH	Water Hardness (mg/L)
Iodine	12.5 to 25	30	≥ 24	$\leq 5^a$	N/A
Chlorine ^b	25	7	49	≤ 10	N/A
	50	7	24	≤ 8	N/A
	50	7	38	10	N/A
	100	10	13	≤ 10	N/A
Quaternary Ammonium Compounds	200	30	≥ 24	N/A	≤ 500

a - or a pH no higher than the level for which the manufacturer specifies the solution is effective.

b – any of the four sets of conditions specified may be used

N/A – not applicable

Occurrence of Bacteria in Dishcloths used in Restaurants

1.2 Abstract.

Dishcloths used in restaurants and bars (23 restaurants, 14 bars) were collected, and tabletops (10 restaurants) were swabbed, to determine the occurrence of bacteria. Coliforms were isolated from 89.2% of dishcloths and 70% of tabletops. *Escherichia coli* was isolated from 54.1% of dishcloths and 20% of tabletops. The numbers of heterotrophic plate count bacteria (HPC) and coliforms were significantly higher in bars than in restaurants. The levels of HPC found in dishcloths were 25-fold and coliforms were 60- to 120-fold lower than the levels found in home dishcloths reported in previous studies. The numbers recovered from restaurant tabletops were also lower than those from household kitchen countertops. The most commonly isolated genera from dishcloths in restaurants and bars differed from those in homes. The numbers found for HPC on restaurant tabletops were 45-fold greater after cleaning than prior to cleaning. There were also a 19-fold greater number of coliforms and twice as many *E. coli*. Therefore, although the mandatory use of sanitizers in restaurants and bars may have reduced contamination levels and caused a shift in the microbial populations present in food service establishments, the implication of dishcloths in contamination of tabletops through cleaning suggests that current monitoring of linen sanitation solutions might be inadequate.

1.3 Introduction

In the United States each year, an estimated 76 million cases of foodborne gastroenteritis occur, with 325,000 hospitalizations and 5,194 deaths (Mead et al., 1999). The microbial causes of foodborne illness include viruses, bacteria and parasites, with symptoms ranging from mild gastroenteritis to life-threatening neurologic, hepatic and renal disease (Mead et al., 1999). Because food is transported to consumers through long chains of industrial production, processing and distribution, numerous circumstances allow for contamination along the way, and existing regulations may not be sufficient to prevent illness. It is helpful to understand the mechanisms by which such contamination occurs in order to reduce the risk of foodborne illness (Tauxe, 1997).

Epidemiological surveillance is important in determining the types of foods responsible in outbreaks, the populations at risk, the circumstances that lead to food contamination, and the growth/survival of foodborne pathogens (Käferstein, 1997). Data collected by the US Food and Drug Administration (FDA) from nearly 900 institutional food service establishments, restaurants, and retail food stores identified improper holding times and temperatures, contaminated equipment/cross contamination, inadequate cooking and poor personal hygiene as risk factors for foodborne disease (Food and Drug Administration, 2000). Between 1988 and 1997, restaurants (2,158) were the most significant sources of foodborne outbreaks, followed by residences (1,032) (Bean et al., 1996; Olsen et al., 2000).

When contaminated cloths come into contact with fingers or surfaces, microorganisms are readily transferred. This may represent a risk if there is a subsequent

contact with food (Scott and Bloomfield, 1990b). Studies in domestic kitchens indicate that wet cloths are important elements in such cross contamination. In one study, cleaning cloths impregnated with a quaternary ammonium disinfectant were compared to cleaning cloths used with a detergent (Scott and Bloomfield, 1993). Some of the cloths used with detergent became heavily contaminated within three hours of use. Following use of these cloths for surface cleaning, both, the surface and the cloths became more heavily contaminated, which suggests that cross contamination had occurred between cloths and surfaces. After the quaternary ammonium-impregnated cloths were used for cleaning, a significant reduction in contamination on food preparation surfaces and cloths was found (Scott and Bloomfield, 1993).

Enriquez et al. (1997b) analyzed sponges and dishcloths from household kitchens in the United States. *Pseudomonas spp.* were the most commonly isolated bacteria. Presumptive *Staphylococcus aureus* and *Salmonella spp.* were isolated with similar frequencies for cellulose sponges and dishcloths. Several other *Enterobacteriaceae* were also isolated. Total and fecal coliform were present in large numbers in contaminated cleaning materials, sometimes reaching levels greater than 10^8 colony-forming units (cfu)/ml in liquid samples. In a similar study of cellulose and natural fiber sponges (loofahs), *S. aureus*, *aeromonas spp.*, *Pseudomonas spp.*, *Enterobacteriaceae* and *Serratia spp.* were identified (Chaidez and Gerba, 2000). These findings, as well as the recovery of large numbers of enterobacteria from draining boards (Scott et al., 1982), sinks, and dishcloths in household kitchens (Rusin et al., 1998; Scott et al., 1982) suggests that dishcloths may act as both, reservoirs and disseminators of microbial contamination.

In the current study, the occurrence of heterotrophic plate count (HPC) bacteria, total coliforms, and *Escherichia coli* on tabletops and dishcloths (used to wipe down tabletops) in public restaurants and bars were determined. In addition heterotrophic bacterial isolates were identified. The purpose of this study was to determine if current dishcloths sanitation in restaurants and bars is sufficient to prevent environmental cross contamination and thus the spread of foodborne illnesses in public food service establishments. The microbiological results were also used for comparison with results from previously published household kitchen studies.

1.4 Materials and Methods

Sample collection. Cleaning dishcloths (2025 cm²) were collected from restaurants and bars and placed in Ziploc[™] plastic bags for transport on ice back to the laboratory. Restaurants in the study included fast food chains, pizza, bar & grill, Mexican and Chinese restaurants located in San Francisco, CA, Phoenix, Flagstaff, and Tucson, AZ. Restaurant tabletops were also sampled by swabbing (approximately 156 cm²) with BBL[™] Culture Swabs[™] (Becton Dickinson, Franklin Lakes, NJ, USA) for subsequent transport to the laboratory.

Sample processing. Depending on the latent moisture content of the cloth, 75 to 100 ml of Lethen neutralizing broth (Difco Laboratories, Detroit, MI) was added to the dishcloths in the Ziploc[™] bags. The bags were squeezed to distribute the neutralizer

liquid throughout the cloths. After 5 minutes of manual compression, liquid was wrung from the cloths and collected in sterile tubes.

The tabletop culture swabs were vortexed for 30 seconds and then pliers were used to squeeze the liquid from the swab. This resulted in a sample volume of approximately 0.6 ml. An additional 0.5 ml of Tris-Buffered Saline (TBS) was added to this to bring the final volume per sample to 1.1 ml. In a separate experiment, tabletops were swabbed, wiped down with a dishcloth (by the restaurant staff) and then swabbed once again to determine if cleaning the table had affected bacterial numbers. The swabs were processed as described previously.

HPC bacterial numbers were determined by plating out appropriate serial dilutions from the swab and dishcloth liquids in duplicate onto R2A medium (Difco, Sparks, MD) utilizing the spread plate technique. Agar plates were incubated at 30 °C for five days and then the bacteria were enumerated by counting colony-forming units (CFU). The number of HPC bacteria per square centimeter was then calculated for each sample. Total coliforms and *E. coli* were enumerated using Colilert Quanti-Trays™ (IDEXX Laboratories, Inc. Westbrook, ME) as per the manufacturer's instructions.

Species identification. For the detection of *Listeria monocytogenes*, 1.0 ml of each dishcloth sample was used to inoculate UVM Modified Listeria Enrichment Broths (Difco Laboratories, Detroit, MI) and incubated for 24 h at 30 °C in a dry bath. From turbid UVM broth samples, 0.1 ml volumes were transferred to selective enrichment Fraser Broth (Difco Laboratories, Detroit, MI) and incubated at 35 °C for 24 to 48 h.

After incubation, 0.1 ml from the esculin-positive samples was placed on the selective chromogenic medium RAPID'L. mono (BIO-RAD, Hercules, CA) using the spread plate technique and incubated for an additional 24 to 48 h at 35 °C.

Three disparate colonies from each R2A agar plate were also subcultured on Tryptic Soy Agar (TSA; Difco, Sparks, MD) plates using the streak for isolation method. The pure culture was then transferred to McConkey Agar (Difco, Sparks, MD) plates, Gram-stained and further characterized using the Oxidase and Catalase tests. Isolated colonies from the TSA plates were also resuspended in inoculating fluid (BIOLOG, Inc. Hayward, CA) to turbidity approximately equivalent to Gram-negative and Gram-positive BIOLOG turbidity standards (40-60% transmittance) and then used to inoculate BIOLOG MicroPlates™ (BIOLOG, Inc., Hayward, CA) as per the manufacturer's instructions. The plates were incubated for 24 h at 35 °C. The results were manually analyzed using the BIOLOG MicroLog 1 System (Program Version 4.20).

Statistical Analysis. A Student's t-test was used to compare the bacterial counts recovered from dishcloths in restaurants and bars. Geometric means were used to report the results and were utilized in the statistical analyses. Geometric means were utilized for all bacterial counts because of the presence of outlying data values. Similar studies conducted in household kitchen environments have also employed geometric means (Chaidez and Gerba, 2000; Enriquez et al., 1997b; Rusin et al., 1998).

1.5 Results

HPC, total coliform and *E.coli* bacteria. Geometric means (GM) of approximately 1.9×10^7 cfu /cloth of heterotrophic plate count bacteria (range of 8.5×10^2 to 8.5×10^{10}), 2.2×10^5 cfu /cloth of total coliform bacteria (range of 70 to 1.0×10^{11}) and 1.2×10^2 cfu /cloth of *E. coli* (range of 2.3 to 1.1×10^6) were isolated from dishcloths in restaurants and bars (Fig.1.5.1). Total coliforms were found in 89.2% of the dishcloths sampled (7.6×10^5 cfu /cloth) and *E. coli* in 54.1% of dishcloths (1.9×10^3 cfu /cloth).

A geometric mean of 2.2×10^4 cfu for heterotrophic plate count bacteria (range of 8.3×10^2 to 2.4×10^7), 15.0 cfu for total coliform bacteria (range of 1.0 to 1.2×10^7) and 1.4 cfu for *E. coli* (range of 1.0 to 27.0) were isolated from swabs of tabletops in restaurants (Fig.1.5.1). These numbers represent the bacteria found on the entire surface swabbed (approximately 156 cm^2). Total coliforms were found on 70% of tabletops (40.8 cfu/ 156 cm^2) sampled and *E. coli* was found on 20% of tabletops (5.2 cfu / 156 cm^2).

The levels of bacteria found in dishcloths from bars were higher than those found in dishcloths from restaurants (Fig. 1.5.2). In dishcloths from restaurants, there were approximately 7.7×10^6 cfu/cloth of HPC bacteria, 2.1×10^4 cfu/cloth of total coliforms, and 3.7×10^1 cfu /cloth of *E. coli*. Figures for dishcloths from bars were approximately 8.7×10^7 , 1.0×10^7 , and 8.7×10^2 cfu/cloth of total bacteria, total coliforms, and *E. coli*, respectively. These differences were significant ($P \leq 0.05$) for HPC bacteria and total coliforms, but not for *E. coli*.

Figure 1.5.1 Bacterial levels found in dishcloths and on tabletops in restaurants and bars

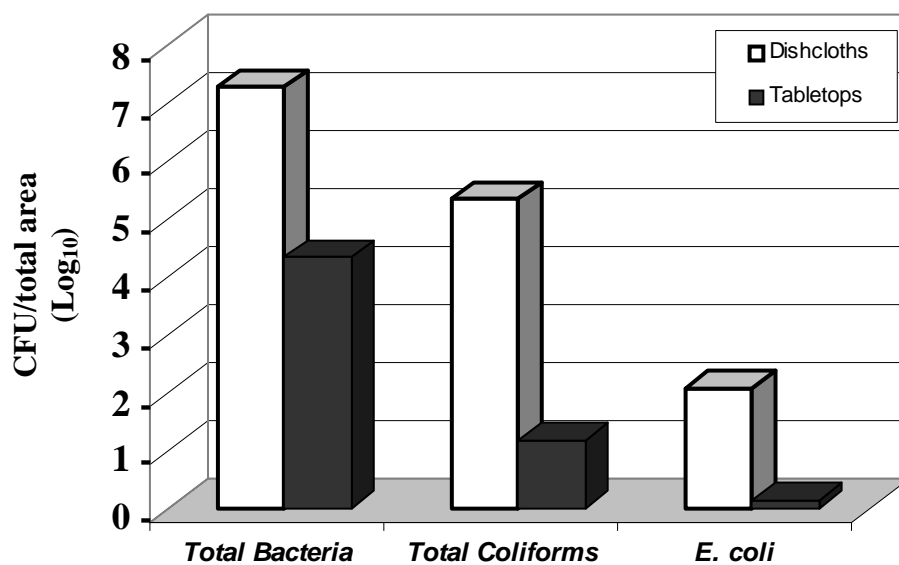
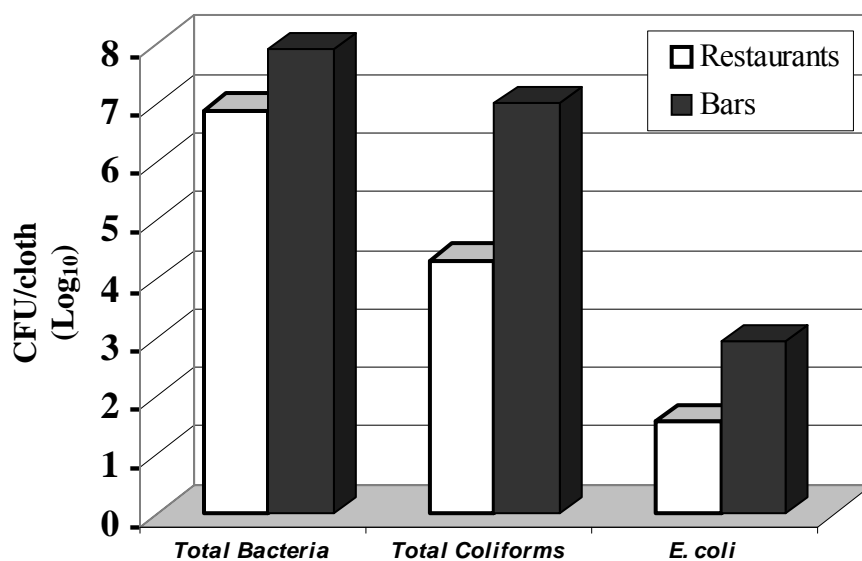


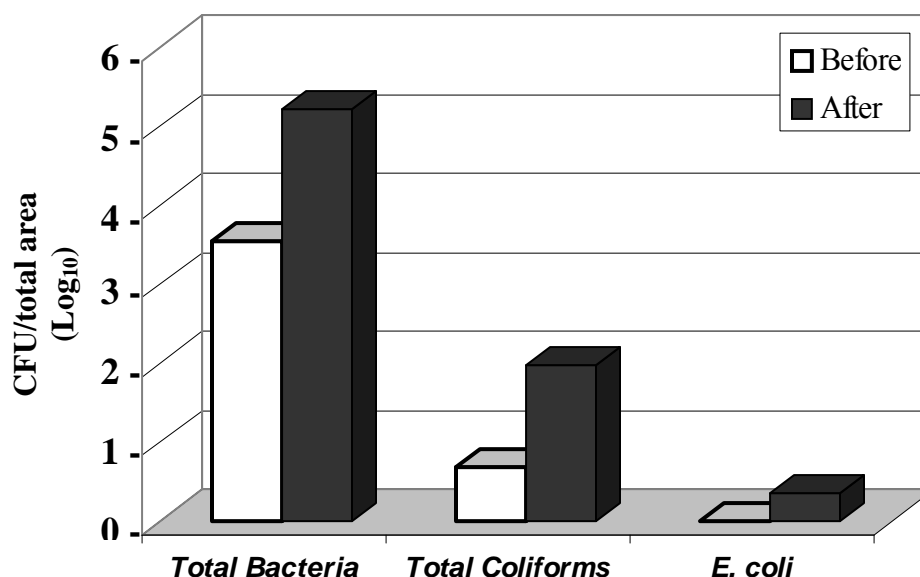
Figure 1.5.2 Comparison of bacterial levels in dishcloths from restaurants and bars.



Greater numbers of bacteria were found on tabletops that had been cleaned with a dishcloth than before cleaning (Fig.1.5.3). Approximately 3.56×10^3 cfu/156 cm² heterotrophic plate count bacteria were found before cleaning. This number increased to 1.6×10^5 cfu/156 cm² (45-fold increase) after the tables were wiped down with a dishcloth. Likewise, the numbers increased for total coliforms (4.9 to 92.2 cfu/156 cm²) and *E. coli* (< 1 to 2.3 cfu/156 cm²) following cleaning.

Bacterial species identification. No isolates of *Listeria monocytogenes* were recovered from dishcloths (0/37) in restaurants and bars; however, *Listeria innocua* was found in 9/37 of the dishcloths (24.3%). A list of bacterial species recovered from dishcloths is shown in Table 1.5.1. The other most commonly isolated species were *Raoultella* (*Klebsiella*) *terrigena* (18.9% frequency), *Pseudomonas maculicola* (16.2%), *Pseudomonas putida* (16.2%), *Pseudomonas fluorescens* (8.1%) and *Ralstonia* (*Pseudomonas*) *pickettii* (8.1%), and *Enterobacter cloacae* (8.1%). The most common genera isolated (Table 1.5.2) were *Pseudomonas* (6 species, 21 isolates, 56.8% frequency), *Klebsiella* (4 species, 10 isolates, 27.0% frequency), *Listeria* (1 species, 9 isolates, 24.3% frequency), *Enterobacter* (5 species, 8 isolates, 21.6% frequency) and *Staphylococcus* (4 species, 4 isolates, 10.8% frequency). *Staphylococcus aureus* was found in 1/37 dishcloths (2.7% frequency).

Figure 1.5.3 Bacteria found on tabletops before and after cleaning in restaurants.



1.6. Discussion

Self-disinfecting sponges, which are colonized by lower numbers of bacteria in comparison to regular sponges, reduce the transfer of total and fecal coliform bacteria to surface and to hands (Enriquez et al., 1997a). Self-disinfecting cloths are often improperly used, causing neutralization of the disinfectant (Scott and Bloomfield, 1993). The use of self-disinfecting clothes is therefore not likely to be a viable option for public food service establishments.

The FDA-approved chemical sanitizers for linens in restaurants and bars, and the specific conditions for their use, are listed on Table 1.1.2. Other sanitizers are also allowed so long as they are used in accordance with the manufacturer's use directions included in the labeling (Anonymous. 2005). The purpose of these sanitizer solutions is to

Table 1.5.1 Bacterial species isolated from dishcloths in restaurants and bars

Species	# Positive	Frequency (%)
<i>Listeria inocua</i>	9/37	24.3
<i>Raoultella (Klebsiella) terrigena</i>	7/37	18.9
<i>Pseudomonas maculicola</i>	6/37	16.2
<i>Pseudomonas putida</i>	6/37	16.2
<i>Pseudomonas fluorescens</i>	3/37	8.1
<i>Ralstonia (Pseudomonas) pickettii</i>	3/37	8.1
<i>Enterobacter cloacae</i>	3/37	8.1
<i>Enterobacter agglomerans</i>	2/37	5.4
<i>Ralstonia (Pseudomonas) solanacearum</i>	2/37	5.4
<i>Cellulomonas hominis</i>	2/37	5.4
<i>Stenotrophomonas maltophilia</i>	2/37	5.4
<i>Acinetobacter calcoaceticus</i>	2/37	5.4
<i>Pseudomonas syringae</i>	1/37	2.7
<i>Klebsiella oxytoca</i>	1/37	2.7
<i>Klebsiella pneumoniae</i>	1/37	2.7
<i>Klebsiella spp.</i>	1/37	2.7
<i>Enterobacter aerogenes</i>	1/37	2.7
<i>Enterobacter asburiae</i>	1/37	2.7
<i>Enterobacter sakazakii</i>	1/37	2.7
<i>Staphylococcus aureus</i>	1/37	2.7
<i>Staphylococcus piscifermentans</i>	1/37	2.7
<i>Staphylococcus sciuri</i>	1/37	2.7
<i>Staphylococcus wameryi</i>	1/37	2.7
<i>Serratia marcescens</i>	1/37	2.7
<i>Serratia rubidaea</i>	1/37	2.7
<i>Kluyvera ascorbata</i>	1/37	2.7
<i>Kluyvera cryocrescens</i>	1/37	2.7
<i>Microbacterium arborescens</i>	1/37	2.7
<i>Microbacterium testaceum</i>	1/37	2.7
<i>Aeromonas veronii</i>	1/37	2.7
<i>Bacillus mycoides</i>	1/37	2.7
<i>Bacillus subtilis</i>	1/37	2.7
<i>Brevundimonas vesicularis</i>	1/37	2.7
<i>Buttauxella izardii</i>	1/37	2.7
<i>Chryseobacterium gleum</i>	1/37	2.7
<i>Comamonas terrigena</i>	1/37	2.7
<i>Corynebacterium thomssenii</i>	1/37	2.7
<i>Dermobacter hominis</i>	1/37	2.7
<i>Escherichia vulneris</i>	1/37	2.7
<i>Herbaspirillum seropedicae</i>	1/37	2.7
<i>Pantoea punctata</i>	1/37	2.7
<i>Paucimonas lemoignei</i>	1/37	2.7
<i>Rhanella aquatilis</i>	1/37	2.7
<i>Roseomonas genomospecies</i>	1/37	2.7

sanitize the cloths after they have been contaminated through use. Sanitization is the cumulative effect of treatments that results in at least a 5- \log_{10} (99.999%) reduction in representative disease microorganisms of public health importance (Anonymous, 2005). The FDA's federal food code recommends that linens used in restaurants and bars to wipe down food service areas be soaked in one of these approved sanitizers under the conditions specified in Table 1.1.2 (Anonymous, 2005). As of September 2004, the federal food code had been adopted by 45 states and one territory and was in the process of being adopted by several others. The FDA's recommendations have therefore been mandated by regulatory agencies in most states (Anonymous, 2005).

Table 1.5.2 Frequency (%) of most commonly isolated bacterial species in dishcloths/cleaning utensils

Species	Restaurants and bars ^a	Household Kitchens	
		Study 1 ^b	Study 2 ^c
<i>Pseudomonas</i> spp.	56.8	31.0 - 38.1	31.8
<i>Enterobacter</i> spp.	21.6	14.3 - 20.7	4.8
<i>Klebsiella</i> spp.	27.0	0	0
<i>Listeria</i> spp.	24.3	ND ^d	ND ^d
<i>Salmonella</i> spp.	0	13.8 - 15.4	9.8
<i>Staphylococcus aureus</i>	2.7	18.6 - 20.0	> 60.0
<i>Aeromonas hydrophila</i>	0	0	19.5

a – present study included only dishcloths

b – study included both dishcloths and sponges (Enriquez et al. 1997b)

c – study included both sponges and loofahs (Chaidez and Gerba, 2000)

d – presence not determined.

As soiled cloths are added to the sanitizing liquid, organic material in the cloths creates a demand on the sanitizer itself. It may also cause changes in pH and water hardness that will decrease the sanitizer's effectiveness or even neutralize the solution. The sanitizing solution should therefore be checked regularly and replenished/refreshed. The temperature of the sanitizing solution may also drop below the recommended minimum, thereby reducing its efficacy. Food service establishments often do not routinely monitor the quality of the sanitizer dip during use, and restaurants and bars therefore often fail to meet these sanitization criteria.

In the present study, bacterial levels found in dishcloths from bars were consistently higher than dishcloths used in restaurants. One possible explanation is that cloths from bars do not become visibly soiled as quickly as those from restaurants and therefore are not sanitized as frequently. Also, workers in bars are perhaps less aware or concerned about the need for proper sanitation of cloths than are restaurants workers. Cloths in bars are usually used to wipe up liquid spills rather than foods. If they are not sanitized as frequently because of lack of visible soiling and/or worker complacency, this provides a moist environment in which bacteria are able to survive for extended periods (Chmielewski and Frank, 1995; Frank et al., 1992).

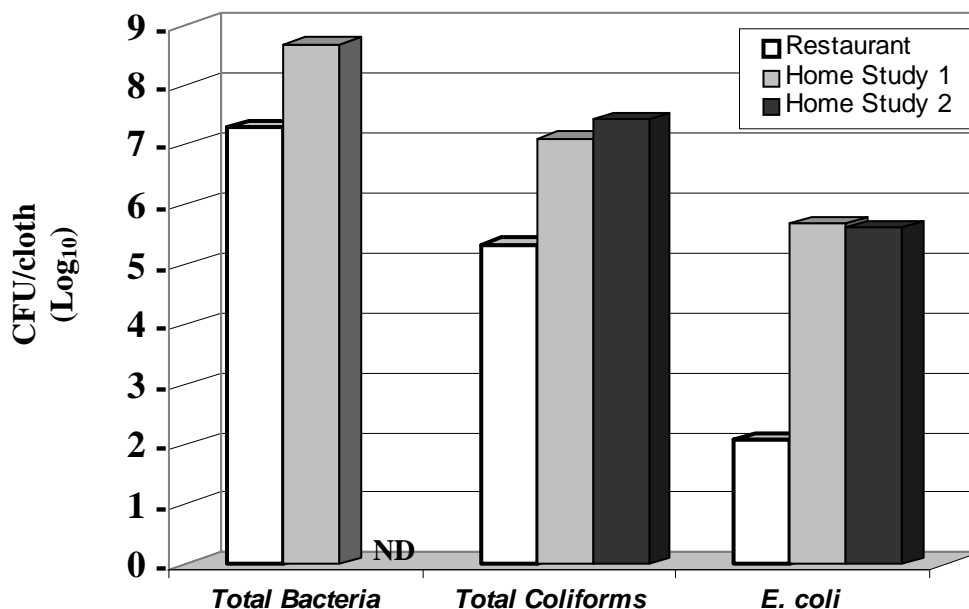
The levels of heterotrophic bacteria, total coliforms and *E. coli* found in dishcloths from restaurants and bars were compared to the levels found in dishcloths in homes, as reported in previous studies (Fig. 1.5.4). The number of HPC bacteria was approximately 25-fold lower in dishcloths from restaurants and bars than in those from homes (Rusin et al., 1998). Likewise, the number of total coliforms was approximately

60- to 120-fold lower in dishcloths from restaurants/bars (Enriquez et al., 1997b; Rusin et al., 1998). The number of *E. coli* was also significantly (3400- to 4000-fold) lower (in comparison to the number of fecal coliforms) (Enriquez et al., 1997b; Rusin et al., 1998). The number of *E. coli* would be expected to be lower than the number of fecal coliforms present; however, the other differences between HPC and coliform bacterial counts could be due to the mandatory use of sanitizers and a great frequency of cleaning in restaurants and bars. One should note that the Colilert assay used to determine the number of total coliforms in our study also varied from the mEndo plate counts utilized in the household studies (Enriquez et al., 1997b; Rusin et al., 1998). Thus, some portion of this discrepancy could be due to the use of different methods.

The total number of bacteria, coliforms and *E. coli* (vs. fecal coliforms) found on restaurant tabletops were also lower (2-, 9- and 12-fold, respectively) than those found on household kitchen countertops (Rusin et al., 1998). This was, again, possibly due to the required use of detergents/sanitizers in restaurants. Total bacteria found on tabletops after cleaning was 45-fold greater than before cleaning, perhaps implicating the dishcloths in tabletop contamination. This was most likely due to the inadequate sanitization of the linens used to wipe down tables.

Listeria monocytogenes was not found in any of the dishcloth samples; however, *Listeria innocua* was present in 24.3% of the dishcloths tested. The presence of another *Listeria* species could indicate that conditions may allow for contamination by and persistence of *L. monocytogenes*.

Figure 1.5.4 Comparison of bacterial levels^a in dishcloths
From restaurants/bars and homes^b.



a – The number of *E. coli* found in restaurants/bars was compared to the number of fecal coliforms in homes.

b – Study 1 (Rusin et al. 1998); Study 2 (Enriquez et al. 1997^b).

ND – not determined

Although many of the bacterial isolates identified were similar for both restaurants/bars and homes, *Pseudomonas* spp. and *Klebsiella* spp. were more prevalent in restaurants and bars whereas *Salmonella* spp., *Staphylococcus aureus* and *Aeromonas hydrophila* were more prevalent in homes (Table 1.5.2). *Salmonella* spp. which may be isolated from raw chicken and eggs, although commonly found in dishcloths used in household kitchens where raw foods are handled, are not likely to be found in dishcloths used to wipe down tabletops in restaurants and bars where cooked foods are generally the only food present. However, the other species differences noted suggest a species shift

between the microbial populations, because presumably the original microbial populations should be similar in both environments. This is also possible due to the mandatory and regular use of sanitizers in restaurants and bars.

Although this study was fairly small, it raises several interesting questions. For instance, although the bacterial numbers found in food service establishments were lower than the number found in homes, considerable numbers of coliforms and *E. coli* were still present. This could represent a danger to the public, especially for populations at risk including the very young, the elderly and the immunocompromised. Also, because the bacterial numbers found on tabletops after wiping with a cloth were higher than the numbers prior to cleaning, the use of such cloths in restaurants and bars could contribute to contamination of surfaces and to the spread of potentially harmful bacteria. Therefore, more careful monitoring of linen sanitization solutions used by food service establishments such as restaurants and bars might be called for.

Additional note. When dealing with environmental samples, it is expected to find great differences between samples, and therefore large standard deviations. If error bars are used in a graph with this kind of behavior, they would be much bigger than the values plotted in the graph.

CHAPTER 2

RESPIRATORY VIRUSES ON PRODUCE

2.1 Literature Review.

2.1.1 Diseases, physical and chemical properties.

Viruses are ultramicroscopic pieces of RNA or DNA enclosed in a protective protein coat called a capsid or nucleocapsid (Sopayrac, 2002). Some viruses have a lipid envelope surrounding the nucleocapsid (enveloped). The function of the capsid or the envelope is to protect the viral genome while it is extracellular and to promote the entry of the genome into a new susceptible cell (Strauss and Strauss, 2002). Viruses are obligate intracellular parasites whose nucleic acid genomes control and utilize the synthetic capacities of their host cells for replication (Pepper and Gerba, 2004). Viruses replicate by synthesizing multiple copies of their genome and their proteins, which assemble spontaneously within the host cell to form progeny virus particles (Meissner and Schaechter, 1989).

Most human viruses cause some form of disease in their hosts, although some of these pathological conditions affect only a small subset of infected humans, or are so mild as to be virtually undetectable. The diseases that viruses cause are the consequences of the way each virus has chosen to solve the problems of reproduction, spread, and evasion of the host's immune system (Sopayrac, 2002). Even though newer infectious agents have been identified, the viruses that have emerged during the past three decades

remain the major contributors to the problem of respiratory infections, the leading cause of acute morbidity in the community (Monto, 1995).

Viral respiratory infections (VRIs) represent the most widespread infectious disease in the United States (Monto, 1995) and are associated with considerable direct and indirect costs, as well as impaired quality of life (Bertino, 2002). Recent data suggests that the total cost of VRIs in the U.S. is, conservatively, \$25 billion in direct and indirect costs. Approximately 500 million episodes of VRIs that occur each year results in 84 million ambulatory office visits for acute respiratory infection (ARIs), 76 million of which are managed by primary care providers (90%), 25 million visits for upper respiratory infection (URIs), 13 million visits for otitis media, 14 million visits for pharyngitis, 13 million visits for bronchitis, and 11 million visits for sinusitis (Bertino, 2002).

Respiratory disease is associated with a large number of viruses including rhinoviruses, coronaviruses, human parainfluenza viruses (HPIV), respiratory syncytial virus (RSV), influenza virus, and adenovirus. When they infect the upper respiratory tract, they can cause acute viral rhinitis or pharyngitis (common cold); when the primary site of infection is the lower respiratory tract, they can cause laryngotracheitis (croup), bronchitis, or pneumonia (Strauss and Strauss, 2002).

The mode of transmission of respiratory viruses is influenced by the type of virus. For example, fragile enveloped viruses such as influenza and parainfluenza virus, respiratory syncytial virus (RSV), and coronaviruses are usually transmitted by close contact with infected individuals, whereas the more stable non-enveloped viruses such as

adenoviruses and rhinoviruses are commonly transmitted by self-inoculation via contaminated hands (Rusin et al., 2000). Infection begins when contaminated air is inhaled or when virus is present in mucosal secretions (e.g., on doorknobs or on a companion's hands), and the virus is transferred to mucosal surfaces in the nose, mouth, or eyes (Strauss and Strauss, 2002). Table 2.1.1 shows the respiratory viruses and serotypes in each genus that are significant causes of viral respiratory illness in humans.

Table 2.1.1 Human respiratory viruses and serotypes

Virus	Serotypes
Influenza viruses	A, B, C
Respiratory syncytial virus	
Parainfluenza viruses	1, 2, 3, 4
Rhinoviruses	100
Adenoviruses	9
Coronaviruses	229E, OC43
Others: herpes simplex, Epstein-Barr, Enterovirus, Measles	

(Couch and Englund, 1997)

Rhinoviruses are the most common pathogen causing VRIs and account for approximately 50% of colds on an annual basis (Hayden, 2002). Rhinovirus infection is very efficient and proceeds very rapidly. The incubation period for infection is 8 to 12 h. One of the early symptoms reported is a scratchy throat, followed by sneezing, nasal

discharge, nasal obstruction, sore throat, hoarseness, cough, headache, myalgia, fever, and chills. The mean duration for rhinovirus colds is 7.5 days (Gwaltney, 2002). Rhinoviruses are not only the primary etiologic agent in common colds, they also cause complications involving the upper and lower respiratory tract. These include otitis media (particularly in children), sinusitis, and exacerbations of asthma and other forms of airway disease, such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (Hayden, 2002). In addition, rhinovirus can cause serious lower respiratory disease in certain populations including infants and young children, elderly persons (Greenberg, 2002), and immunocompromised patients (Couch and Englund, 1997).

Rhinovirus is a nonenveloped particle that belongs to the family *Picornaviridae*. The picornavirus virions are composed of 60 copies of each of four different proteins (called VP1-4) that form an icosahedral shell having a diameter of approximately 30 nm. Their genome is a positive-sense, single-stranded RNA molecule of about 8 kb in size. The rhinovirus capsid is loosely packed, accounting for rhinovirus' greater density and its susceptibility to inactivation by exposure to acids (labile at pH 3-5) (Gwaltney, 1997).

The optimal temperature for rhinovirus growth is 33 to 35°C, which corresponds with the normal temperature of the nasal mucosa (Rusin et al., 2000), and may explain why these viruses propagate most efficiently in the upper respiratory tract (URT). At 37°C, virus yields fall to 10-50% of the optimum. Rhinovirus has not been isolated from blood, and does not survive or replicate in the intestinal tract. These may be due to the gut temperature of 37°C, in addition to the gastrointestinal secretions and transit time (Gwaltney, 1997). The rhinovirus RNA polymerase is error prone, so it can use antigenic

drift as a mechanism to stay one step ahead of the adaptive immune system. As a result, over one hundred different strains of rhinovirus are currently circulating in the population (Sompayrac, 2002).

Coronaviruses, whose name comes from the crown-like appearance of the array of spikes around the enveloped virion, are responsible for about 25% of human colds of all age groups, and are spread by the respiratory route. Unlike rhinoviruses, they cause only URT infections but sometimes lower respiratory tract (LRT) as well. Some coronaviruses may cause gastroenteritis in humans. There have been reports of coronaviruses in the stools of people suffering from gastroenteritis (Strauss and Strauss, 2002).

The total number of serological types that infect man has not defined due to the difficulties encountered in isolating human coronaviruses; however, human coronaviruses (HCoV) 229E and OC43 have been known for 30 years (Vallet et al., 2004). HCoV-229E has been recently recognized as a potential agent in nosocomial viral respiratory infections (NVRI) in high risk infants (children and newborns) and hospital staff members (Gagneur et al., 2008). Recently, three distinct human coronaviruses have been discovered, including the etiological agent associated with severe acquired respiratory syndrome (SARS). These viruses, with the exception of the SARS CoV, are likely to be common respiratory viruses and may be responsible for a substantial proportion of respiratory tract disease (Kahn, 2006b).

HCov-NL63 and HCoV-HKU1 were first described in 2004 and 2005, respectively, and have been circulating in humans for a long time. They are frequently found during

URT and LRT infections and have spread worldwide, seemingly preferring the winter season. Their symptoms do not differ greatly from those described for the older coronaviruses (HCoV-229E and HCoV-OC43). HCoV-NL63 and HCoV-HKU1 are likely related to HCoV-229E and HCoV-OC43, respectively (van der Hoek, 2007).

SARS is a rapidly progressing pneumonia that affects all age groups in an epidemic manner. It emerged in southern china in late 2002 and spread rapidly from China to more than 30 countries including Canada, Singapore, Vietnam and Taiwan (Wei-Kung et al., 2004), and to Europe, South America and North America before the global outbreak of 2003 was contained (CDC, 2004; Subbarao et al., 2004). According to the World Health Organization, 8,437 cases of SARS had been identified worldwide as of July 11th, 2003 and 813 patients had died, resulting in an overall mortality rate of 9.6% (WHO, 2003). The syndrome is characterized by fever, chills or rigors, headache, and nonspecific symptoms such as malaise and myalgia, followed by cough and dyspnea. The disease progresses to acute respiratory distress syndrome, requiring intensive care and mechanical ventilation for more than 20% of the patients (Subbarao et al., 2004).

The family *Coronaviridae* (recently classified together with the *Arteriviridae* in the order *Nidovirales*) is composed of two genera, the genus *Coronavirus* and the genus *Torovirus*. The coronavirus virion is enveloped, it has a positive non-segmented single stranded RNA. It is 120-160 nm in size and has the largest viral genome known of 30-kb. It contains three major proteins, the nucleocapsid protein (N), the membrane glycoprotein (M), and the large spike glycoprotein (S), which form the distinctive projections of the virus. The virus is more stable at pH 6.0 and low temperature appears to protect it against

varied relative humidity (Monto, 1997). Gundy et al. (2009) demonstrated that the transmission of coronavirus in the aqueous environment is low due to the fact that coronaviruses are more rapidly inactivated in water and wastewater at ambient temperature. They found that only 10 days are required to result in a 99.9% reduction of coronavirus in filtered tap water at room temperature.

Human Parainfluenza viruses (HPIVs) are second to RSV as a common cause of lower respiratory tract (LRT) disease (pneumonia, bronchitis, and bronchiolitis) in young children, the elderly, and among patients with compromised immune systems. They commonly reinfect older children and adults to produce upper respiratory tract (URT) illnesses (cold and/or sore throat) (Glezen and Denny, 1997a). Each one of the four HPIVs has different clinical and epidemiologic features. Table 2.1.2 shows the serotype of HPIV and the clinical manifestations they cause. Serological surveys have shown that 90 to 100% of children aged 5 years and older have antibodies to HPIV-3, and about 75% have antibodies to HPIV-1 and HPIV-2. (CDC, 2006).

Table 2.1.2 Human parainfluenza serotypes and their main manifestations.

Types	Disease
1, 2	croup (laryngotracheobronchitis)
3	pneumonia, bronchiolitis
4	infrequently detected, inconsequential

(CDC, 2006)

HPIVs are negative-sense, single stranded RNA viruses that possess fusion and hemagglutinin-neuraminidase glycoprotein (spikes) on their surface (enveloped). The virion size average diameter is between 150 and 300 nm. The virus is unstable in the environment, surviving only a few hours on environmental surfaces, and is easily inactivated with soap and water (CDC, 2006). The viruses are ether sensitive and acid labile. They have the ability to replicate in the respiratory epithelium without deeper invasion. The incubation period is short, between 3 to 6 days, and the virus spreads rapidly to a high percentage of persons in closed populations, which indicates their high degree of infectiousness (Glezen and Denny, 1997a).

Respiratory syncytial virus (RSV), is a contagious virus that can lead to serious health problems, especially for young children and older adults (CDC, 2008). RSV can cause upper respiratory tract (URT) infections such as colds. Most healthy people recover from infection in 1 to 2 weeks; however, when the lower respiratory tract (LRT) is affected, RSV is more severe and is the most common cause of bronchiolitis (inflammation of the small airways in the lungs) and pneumonia in children less than 1 year of age in the U.S and around the world (McIntosh, 1997). In addition, RSV is more often being recognized as an important cause of respiratory illness in older adults, adults with chronic heart or lung disease, or people with immune systems weakened by medical conditions or treatments (CDC, 2008).

When infants and children are exposed to RSV for the first time, 25 to 40% have signs or symptoms of bronchiolitis or pneumonia, and 0.5 to 2.0% require hospitalization,

half of which are less than 8 months of age (CDC, 2008). Approximately 90,000 hospital admissions and 4,500 deaths are associated each year with RSV in the U.S. in both infants and young children (Ginsberg, 1990). RSV is the most common cause of nosocomial infections in pediatric wards and the most important cause of middle ear infection in children. Immunity following infection is incomplete and reinfection is common in children and adults. Reinfection tends to produce less severe disease though (McIntosh, 1997). RSV is often introduced into the home by school-aged children who are infected and have a mild URT infection, such as a cold. RSV can be rapidly transmitted to other members of the family, often infecting about 50% of other household members. In the U.S. RSV infections generally occur during fall, winter, and early spring (CDC, 2008).

RSV belongs to the family *Paramixoviridae*, as the only member of the genus *Pneumovirus*. RSVs are spherical, medium-sized (120-200 nm), enveloped particles that contain a lipoprotein coat and a linear, negative-sensed RNA genome (Ginsberg, 1990).

RSV does not survive well in the environment. If suspended in a protein-free medium at 4°C, 90 to 99% of their infectivity is lost within 4 hours. Organic solvents and detergents rapidly inactivate them by dissolving their lipid envelopes (Ginsberg, 1990).

Influenza Virus causes an acute and potentially serious infection that is estimated to cause 36,000 deaths and more than 200,000 hospitalizations annually in the United States (Rothberg et al., 2008). The economic burden of annual influenza epidemics based on

2003 data was estimated at \$87.1 billion and included hospitalization days, deaths, outpatient visits, plus other indirect costs (Poland and Johnson, 2008).

Classic influenza is generally an uncomplicated and self-limited illness, characterized by respiratory as well as constitutional signs and symptoms, such as a nonproductive cough, high fever, chills, headache, sore throat, myalgia, and malaise (Poland and Johnson, 2008). Additional symptoms unique to children include abdominal pain, diarrhea, and vomiting. Influenza infection can lead to serious sequelae, including secondary bacterial pneumonia, sinusitis, bronchitis, and myocarditis, as well as croup, bronchiolitis, and acute otitis media in children. Influenza can also exacerbate underlying clinical conditions and trigger acute myocardial infarction or stroke and increase the rate of death from coronary heart disease (Poland and Johnson, 2008).

Influenza viruses are divided into types A, B, and C. Influenza virus type A is further classified into many subtypes according to host of origin, year, and geographic location of first isolation. All three types of virus infect man and cause disease, but influenza A represents the most serious human pathogen because it causes very large, recurrent epidemics with significant mortality (Strauss and Strauss, 2002).

The major factor responsible for the recurring nature of influenza epidemics is antigenic variation of the surface glycoproteins of influenza viruses. These changes lead to renewed susceptibility of persons infected previously so that reinfection and illness may occur. Types B and C appear to be unique pathogens of man and demonstrate less antigenic diversity than type A (Glezen and Couch, 1997b).

High risk patients aged 45 to 64 years have a risk equivalent to that of all patients aged more than 65 years. Because of the high incidence of hidden cardiopulmonary disease in older persons, the Advisory Committee on Immunization Practices has extended its vaccination recommendation to include all persons more than 50 years old. The most recent expansion calls for universal vaccination of all children through 18 years of age and the adoption of a new paradigm that includes a much longer vaccination period, starting in the autumn and continuing through the entire influenza season (October to May) to protect the large number of at-risk persons (Poland and Johnson, 2008).

Influenza A virus, influenza B virus and influenza C virus belong to three genera of the family *Orthomyxoviridae*. Their approximate size is 80 to 120 nm and they possess a lipid envelope with a genome consisting of eight segments of negative-sense, single-stranded RNA (influenza C has only 7 segments) (Strauss and Strauss, 2002). Influenza A and B have two surface glycoproteins projecting from the envelope, hemagglutinin (HA) and neuraminidase (NA), whereas influenza C has only one, called HE protein that exhibits hemagglutination and esterase activity (Glezen and Couch, 1997b).

Influenza A virus can be transmitted most efficiently through aerosols. It has been demonstrated that influenza A virus remains infectious for at least 1 hour in aerosols at room temperature; however, it is probable that influenza is also transmitted by direct contact, but its spread among the population and the overall attack rates are low (Glezen and Couch, 1997b).

Adenoviruses most commonly cause respiratory illnesses; however, depending on the infecting serotype, they may also cause various other illnesses, such as gastroenteritis, conjunctivitis, cystitis, and rash illness. Symptoms of respiratory illness infection range from the common cold syndrome to pneumonia, croup, and bronchitis (CDC, 2005). Human adenoviruses replicate primarily in the upper respiratory tract (URT) or in the gut. Some replicate well in both while others express a tropism for one or the other. Spread of the virus is by a respiratory route, by an oral/fecal route, and occasionally as a waterborne infection (Strauss and Strauss, 2002).

Fifty-one human adenoviruses have been distinguished. They have been divided into six subgroups (A to F), on the basis of properties of the DNA, serological cross-reactions in a hemagglutination-inhibition assay, and oncogenicity (Foy, 1997). Most adenovirus human disease is associated only with one-third of adenovirus types. Although many adenovirus infections are asymptomatic, these viruses can cause acute respiratory disease (ARD) (types 1 to 7, 14, and 21), conjunctivitis (types 3, 7, 8, 11, 14, 19, and 37), acute hemorrhagic cystitis (types 11 and 21), ARD of military recruits (types 3, 4, 7, 14, and 21), and gastroenteritis (types 31, 40 and 41) (Horwitz, 1996).

About 5% of respiratory disease in children under 5 years old and 3% in adults is due to adenovirus infection. The disease is usually mild, but types 4 and 7 have caused epidemics of ARD in 80% of the recruits in a military unit, and 20 to 40% of these required hospitalization. The stress and crowding seems to potentiate the illness (Strauss and Strauss, 2002).

Adenovirus belongs to the family *Adenoviridae*. They are medium sized (90-100 nm), nonenveloped, icosahedral viruses containing double-stranded DNA. They are unusually very stable to chemical and physical agents and adverse pH conditions, allowing for prolonged survival outside the body (CDC, 2005). Contaminated inanimate surfaces (fomites) may play a significant role in adenovirus transmission because of its stability to drying. At room temperature, adenovirus 2 survives for 8 and 12 weeks at low (7%) and high (96%) relative humidity (Horwitz, 1996), being more resistant than poliovirus 2. This property may be associated with the double-stranded nature of their DNA, which, if damaged, may be repaired by the host cell DNA repair mechanisms (Rusin et al., 2000).

Human Metapneumovirus (HMPV). In nearly half of upper respiratory tract (URT) illnesses in children, an infectious cause cannot be determined. The etiology of a majority of lower respiratory tract (LRT) illnesses is thought to be viral, yet in only 40% of cases can a viral agent be identified, even with use of state-of-the-art genomic amplification methods. These observations suggest that previously unknown pathogens may be circulating and may be responsible for a substantial proportion of respiratory tract disease (Kahn, 2006a). Human metapneumovirus (HMPV) was first identified in 2001 in the Netherlands from archived respiratory cultures collected from infants and young children in whom other pathogens could not be isolated (van den Hoogen et al., 2001). Studies have demonstrated that HMPV infection induces incomplete immunity and reinfection occurs later at all ages so repeated illness with childhood viruses are common in all

adults, and along with RSV and influenza virus, HMPV cause a significant disease burden in older adults, especially among those hospitalized during the winter months with respiratory illness (Walsh et al., 2008). One study reported that 70% of RSV-infected children, who required intensive care in the U.K., were coinfecting with HMPV, suggesting that the disease caused by RSV may be augmented by a concurrent HMPV infection, particularly in otherwise healthy children (Greensill et al., 2003).

Serological studies revealed that by the age of 5, nearly all individuals had evidence of HMPV infection; the seroprevalence of HMPV specific antibody in adults is nearly 100%. The seroprevalence of HMPV-specific antibody in infants <3 months of age is >90%, indicating that maternally derived antibodies are present in young children. The virus has been circulating in humans for at least 50 years, suggesting that the virus did not recently “jump” to the human population from an animal reservoir such as birds (van den Hoogen et al., 2001; Kahn, 2006a).

HMPV is negative-sense, single-strand, enveloped RNA virus classified in the *Paramyxoviridae* family, which is divided into two sub families: *Paramixovirinae* and *Pneumovirinae*. They are closely related to RSV and parainfluenza viruses (Strauss and Strauss, 2002).

2.1.2 Survival of respiratory viruses on the environment: fomites.

Environmental characteristics play an important role in the survival of a virus; however, of much greater significance is the influence of the environment on the route of transmission and on the behavior patterns of the host. The respiratory route is probably the most important method of spread for most common viral diseases of man and is subject to the least effective environmental control (Kaslow and Evans, 1997).

Another aspect that affects the transmission of respiratory viruses is the intensity and method of propulsion of discharges from the mouth and nose via coughing, sneezing and talking, the size of the aerosol droplets created, and the resistance of the airborne virus to desiccation (Kaslow and Evans, 1997). Direct transmission of infection occurs via personal contact such as kissing, touching of contaminated objects (fomites), and direct impingement of droplets containing infectious virus that are easily transmitted over considerable distances (Boone and Gerba, 2005).

Airborne viruses present in sneezing can travel at a speed of around 150 km/hour, so this is a very efficient way of spreading germs (Mullins, 2003). The dispersion of an aerosol depends on wind currents and on particle size. Particles of 6- μm or more are usually trapped in the nose, whereas those between 0.6 and 6.0- μm are deposited on sites along the upper and lower respiratory tracts (Kaslow and Evans, 1997). In still air, a spherical particle 100- μm in diameter requires 10 sec to fall the height of the average room (3 m), particles 40- μm require 1 min, 20- μm particles require 4 min, and 10- μm particles require 17 min. Thus, particles under 10- μm in diameter have a relatively long circulation time in the ordinary room. Research performed by Koeniger around the start

of the 20th century regarding bacterial dissemination by droplets from the mouth during coughing and sneezing showed that the greatest distance to which bacteria were carried was 12.40 meters (Reiling, 2000). Viruses in aerosols eventually settle on environmental surfaces.

Fomites consist of both porous and nonporous surfaces or objects that can become contaminated with pathogenic microorganisms and serve as vehicles in transmission (Boone and Gerba, 2007). For any environmental contamination to be relevant, the virus should not only remain infectious but also persist at a sufficient concentration to enable it to reach the respiratory tract via finger contamination (Thomas et al., 2008). There is growing evidence that contaminated fomites or surfaces play a key role in the spread of viral infections (Boone and Gerba, 2005; Kramer et al., 2006; Boone and Gerba, 2007; Winther et al., 2007). Viruses can be easily spread to the mouth when fomites and hands become contaminated. A small child puts fingers in his/her mouth once every 3 minutes and children up to 6 years average a hand-to mouth frequency of 9.5 contacts per hour (Tulve et al., 2002).

Respiratory viruses that have been detected on surfaces in hospitals, daycare centers, nursing homes, workplace offices and in the home are respiratory syncytial virus, rhinovirus, influenza virus, parainfluenza virus, and coronavirus. The surfaces on which they can survive vary from countertops, desks, phones, computer mouse, doorknobs, toilet handles, to latex gloves, cloth gowns and towels (Boone and Gerba, 2007).

Rhinovirus is the most common respiratory virus known to be easily transmitted by fomites. To assess the transfer of rhinovirus from surfaces during normal daily

activities to fingertips, 15 adults with natural rhinovirus colds stayed overnight in a hotel. Subsequent transfer to fingertips of five healthy subjects was examined by drying 10 μ l of virus-containing mucus from each subject onto light switches, telephone dial buttons and telephone handsets. After an interval of 1 or 18 hours the subject flipped the light switch, pressed the button, and held the handset. Fingertip rinses were tested for the virus. Thirty-five percent of the 150 environmental sites in the rooms were contaminated. Common virus-positive sites included door handles, pens, light switches, TV remote controls, faucets, and telephones. Rhinovirus was transferred from surfaces to fingertips in 18/30 (60%) trials 1 hour after contamination and in 10/30 (33%) trials 18 hours (overnight) after contamination (Winther et al., 2007).

Given that billions of banknotes are exchanged daily worldwide, Thomas et al., (2008) assessed the survival of human influenza virus on banknotes experimentally contaminated with representative influenza virus subtypes at various concentrations. Influenza A viruses survived up to 3 days when they were inoculated at high concentrations. However, the same inoculum in the presence of respiratory mucus showed an increase in survival to 17 days. Authors suggested that the unexpected stability of influenza virus in this nonbiological environment should be considered in pandemic preparedness efforts.

The most common nosocomial pathogens may well survive or persist on surfaces for months and can thereby be a continuous source of transmission if no regular preventive surface disinfection is performed. Kramer et al. (2006) reviewed the persistence of different nosocomial pathogens on inanimate surfaces. Viruses from the

respiratory tract such as influenza virus may persist for 1 to 2 days, respiratory syncytial virus for up to 6 hours, rhinovirus from 2 hours to 7 days, coronavirus for 3 hours, and SARS associated coronaviruses for 72 to 96 hours. The authors identified factors influencing pathogen persistence on surfaces, concluding that overall, a high inoculum of the pathogen in a cold room with high relative humidity will increase the chances for longer persistence.

Rabenau et al. (2005) studied the stability of SARS coronavirus (SARS-CoV) under different conditions, both in suspension and dried on surfaces in comparison to other human-pathogenic viruses including human coronavirus HCoV-229E. In suspension, HCoV-229E gradually lost its infectivity completely whereas SARS-CoV retained its infectivity for up to 9 days. In the dried state, survival times were 24 hours versus 6 days for these viruses.

An outbreak of acute keratoconjunctivitis involving 27 patients occurred in an ophthalmology ward. Adenoviral DNA was detected in four inpatients, one outpatient and one healthcare worker. At a later stage of the outbreak, adenoviral DNA types 37 and/or 3 were detected from almost all environmental instruments and commonly used eye drops, despite thorough disinfection of the environment and enforcement of various infection control measures. The detection rate of adenoviral DNA in environmental swabs was 81%. A second disinfection of the environment reduced the detection rate of adenoviral DNA to 38%. The outbreak ceased after closing of the ophthalmology ward and outpatient consulting room, accompanied by enhanced cleaning of environmental

instruments and the introduction of disposable eye drops for individual patients (Hamada et al., 2008).

To assess the potential role of fomites in the transmission of influenza, the prevalence of influenza A virus on surfaces in day care and home setting was evaluated. 218 fomites from 14 different day care centers and 92 fomites from 8 different homes with children were evaluated. Influenza was detected on 23% of day care fomites during the fall and 53% of fomites sampled during the spring. No influenza was detected on home fomites during the summer. In contrast, influenza was detected on 59% of home fomites sampled during March (Boone and Gerba, 2005).

Pathogens are readily transferred to hands from contaminated fomites and to the mouth from contaminated hands. To determine the transfer efficiency of microorganisms from fomites to hands and the subsequent transfer from the fingertip to the lip, Rusin et al. (2002) collected samples from volunteers' hands after the normal usage of fomites seeded with a pooled culture of bacteria and the PRD-1 phage. After performing activities such as wringing out a dishcloth or sponge, turning on and off a kitchen faucet, cutting up a carrot, making hamburger patties, holding a phone receiver, and removing laundry from the washing machine, they found transfer efficiencies of 38.5 to 65.8% for the phone receiver and 27.6 to 40% for the faucet to hands. When the volunteers' fingertips were inoculated with the pooled organisms and held to the lip area, transfer rates were of 41% and 34% for the bacteria tested, and 34% for PRD-1 virus.

To provide novel information on the occurrence of hygienic markers on public surfaces and identify the relative importance of surfaces where exposure rates may be

highest, 1,061 environmental surfaces were monitored by Reynolds et al. (2005). Samples from shopping, daycare, and office environments, personal items, and miscellaneous public surfaces in four US cities were analyzed for the occurrence of fecal and total coliform bacteria, heterotrophic plate count bacteria, proteins, and biochemical markers for biological substances. Alpha-amylase, one of the biomarkers normally present in human and animal mucosa, saliva, and urine, was found on 15% of the samples analyzed. The five most frequently positive sites were: children's playground equipment, daycare surfaces, public phones, computer keyboards, and vending machines.

In addition, to evaluate the potential transfer and main exposure routes of surface contaminants from public places to hands and other environments, three office environments were monitored after artificial inoculation of common use surfaces with a fluorescent copolymer resin tracer. After touching contaminated surfaces, 86% of office workers transferred the resin tracer to their hands while 82% transferred the resin to additional surfaces in a way similar to the manner contaminated surfaces could spread infectious doses of pathogens to the mouths of exposed individuals following handling (Reynolds et al., 2005).

Insufficient hand washing contributes greatly to the communicable nature of colds and gastrointestinal infections. The United States Centers for Disease Control and Prevention (CDC) states that hand washing is the best way to prevent the spread of infections. Some studies, however, indicate that maintaining an effective hand-washing program in U.S. schools is difficult because many classrooms, even with proper facilities, the time required for 20 to 30 students to perform minimal hand washing (20 to 30

seconds each) would significantly interfere with classroom instruction time (Dyer et al., 2000). In response to the need for hand sanitization in situations where soap and water are not readily available and time is limited, antimicrobial rinse-free hand-sanitizing formulas have been developed. Some of hand sanitizers have several limitations. Nevertheless, sanitizers containing surfactants, allantoin, and benzalkonium chloride (SAB formulation) surpass the FDA performance standards for fast acting, have broad antimicrobial spectrum, display persistence of activity, and are effective under heavy bacterial soil load (Dyer et al., 2000).

Some studies have suggested links between biocides and antibiotic resistance, but there is currently no evidence that it is a significant factor in the development of antibiotic resistance in clinical practice (Bloomfield, 2002). Although it is generally agreed that the cause of antibiotic resistance in clinical practice is the over-prescribing of antibiotics, some scientists have suggested that widespread use of biocides, particularly in consumer products, may be a contributing factor; however, if reducing the number of infections through effective hygiene is important, then it is also important to ensure that biocide use, as an integral part of good hygiene practice, is not discouraged in situations where there is real benefit in terms of preventing infection transmission (Bloomfield, 2002).

2.1.3 Methods for detecting viruses on produce. Typically, a method for the detection of viruses in food involves elution and clarification of the virus from the foodstuff, concentration of the virus particles, and nucleic acid extraction and detection (Croci et al., 2008).

Elution. Detection of virus on fruits and vegetables starts with the elution of the virus particles from the surface of the produce. This is generally done with a basic buffer (pH 7.4 to 9.0) to break the electrostatic and hydrophobic interactions between the produce surface and viruses. Several studies described the different procedures used for the recovery of enteric viruses on produce. Ward et al. (1982) recovered poliovirus and adenovirus from vegetable surfaces by eluting with phosphate buffer saline solution (PBS) at pH 9.0; samples were shaken for 7 to 10 min on a rotary shaker to release the virus. Clarification was made by glass wool filtration pretreated with Maintenance Eagle basal Medium (MEM) with fetal calf serum (CS) to prevent loss by virus adsorption.

In another study, recovery of Hepatitis A Virus (HAV) from experimentally contaminated lettuce and strawberries, was done by repeated pipetting /washing (>25 times) of the contaminated area with 1 ml of PBS (pH 7.6). The virus-containing wash solution was collected and used for virus assay (Bidawid et al., 2000). Dubois et al., (2002) inoculated fruits with poliovirus, HAV and norovirus and placed them in a plastic bag with a filter compartment and soaked in elution buffer (Tris-HCL+glycine+ 3% beef extract, pH 9.5) for 20 minutes at room temperature with constant shaking (Dubois et al., 2002).

For the detection of norovirus on raspberries associated with a gastroenteritis outbreak, Le Guyader et al. (2004) recovered the virus by washing 10 g of berries with glycerin-buffer (pH 9.5) shaken vigorously for 1 minute. The pH was adjusted to 9.5 and the mixture was vortexed for 2 minutes to elute viruses. To quantify HAV from green onions and strawberries, 25 grams of sample was vigorously shaken with PBS (pH 7.4)

for 2 minutes (Shan et al., 2005). Butot et al. (2007) recovered HAV, norovirus, and rotavirus from different types of berries, vegetables and herbs by gentle shaking in elution buffer (glycine + Tris + 1% beef extract, pH 9.5%) for 15 minutes at room temperature.

Clarification. After elution, it is necessary to remove food particles from the eluate containing the viruses in suspension. This clarification is usually performed at high pH to prevent the adsorption of viruses to the fruit or vegetable. Frequently, centrifugation at $14,000 \times g$ or below has been used to clarify the eluate. The pellet containing fruit or vegetable matter is then discarded while viruses remain in the aqueous phase matter (Jaykus, 1997; Croci et al., 2008).

Ward et al. (1982) clarified the eluted virus solution by glass wool filtration pretreated with Maintenance Eagle basal Medium (MEM) with fetal calf serum (CS) to prevent loss by virus adsorption. Dubois et al. (2002) clarified by centrifuging the viral eluate at $10,000 \times g$ for 15 minutes at 4°C to pellet residual vegetal particles; Butot et al. (2007) centrifuged the virus eluant at $3,500 \times g$ for 15 minutes.

Filtration. Filtration through large porosity filters, previously treated for non-adsorption of viruses, has also been used to remove food particles (Dubois et al., 2006). Ward et al. (1982) adsorbed the clarified virus elution to a Filterite pleated cartridge filter and eluted with 3% beef extract pH 9.0. To remove particulate debris, elution buffer was transferred

to a tube containing a nylon cell strainer of 40 μm pore size which was then rinsed with elution buffer (Butot et al., 2007).

Concentration. Considering that contaminated foods may contain very low levels of enteric viruses and the low infectious dose of viruses, the concentration step is very important for realistic detection of enteric viruses in food products (Machado et al., 2009). Virus concentration methods always have to be adapted to be compatible with the eluant used and the type of food that is being analyzed (Croci et al., 2008). For example, juices released from soft fruits might interfere with virus concentration through filtration by clogging the filters. In such a case, precipitation of viruses would be preferable. Viruses can be precipitated by the addition of polyethylene glycol (PEG) to the clarified eluate, which is a compound that reduces the solubility of macromolecules. Salad vegetables are more resistant to disruption by washing treatments than are soft fruits so eluates from salad vegetables can therefore be concentrated by filtration, which is more rapid than concentration by precipitation (Croci et al., 2008).

Ward et al. (1982) concentrated the eluates by organic flocculation and the pellets were dissolved in a buffer solution. Poliovirus was recovered with a mean efficiency of 58% and adenovirus with 55% from lettuce. After the aqueous solution was neutralized to pH 7.0 and supplemented with polyethylene glycol with NaCl and incubated overnight at 4°C, Dubois et al. (2002) concentrated virus in suspension by centrifugation at 10,000 $\times g$ for 2 hours at 4°C. The resulting pellet was suspended on phosphate buffer saline (PBS)

and mixed with a chloroform/butanol solution, and then centrifuged at 12,000 x g for 15 minutes at 4°C.

Dubois et al. (2006) concentrated viruses by filtration using first a membrane filter with a pore size of 5 µm (pre-filter), and then a negatively charged cellulose nitrate membrane filter with a pore size of 3 µm. The viruses were recovered by putting the two filters in a flask containing glycine with 3% beef extract (pH 9.5) and shaken vigorously 3 times for 2 minutes. Viral particles present in the supernatant was collected and clarified by centrifugation and concentrated by the adsorption-elution method using negatively charged membranes. The recovery rate of norovirus on fresh lettuce ranged from 5.2 to 72.3% (Machado et al., 2009).

Ultrafiltration has been described for the concentration of viruses from several foods including fruits and vegetables. In this process, virus is entrapped in a sample because of its molecular size rather than by particle charge. Pores in the membrane, varying from 10 to 100 kDa, permit passage of liquids and low molecular mass particles in solution and exclude viruses and macromolecules (Croci et al., 2008). To develop a rapid, specific, sensitive, and reliable analytical procedure to detect enteric viruses in different types of berries, vegetables and herbs, Butot et al. (2007) adjusted the pH to 7.0 and centrifuged the eluate at 3,500 x g for 15 minutes and the supernatant was concentrated by ultrafiltration. Ultracentrifugation at 100,000 × g can also be used to pellet viruses. This method requires expensive equipment and can be used only with eluates free of vegetable matter (Butot et al., 2007).

Detection. Classical method for detecting viral contamination of foods is by inoculation of cell cultures. This can be costly and time consuming. In addition, food extracts may be cytotoxic to the host cells and some viruses (norovirus for example) do not grow in cell cultures, or grow very poorly (Goyal, 2006).

Virus-specific killing of infected cells or cytopathic effect (CPE) in monolayer cell culture is visible by ordinary light microscopy and allows the determination of the presence or absence of infectious virus. Plaque assays (Ward et al., 1982; Jean et al., 2004; Shan et al., 2005; Dubois et al., 2006) and tissue culture infectious dose 50 (TCID₅₀) assays (Krah, 1991; Dubois et al., 2002; Guevremont et al., 2006; Straub et al., 2007) have been used for the detection of infectious poliovirus and hepatitis A virus (HAV) in different vegetables (Croci et al., 2008).

The polymerase chain reaction (PCR) provides an opportunity to amplify a single specific nucleic acid sequence up to a million-fold and hence provides a sensitive and specific method with a theoretical detection limit of one virus (Croci et al., 2008). This method is readily adaptable to the detection of RNA viruses by preceding the PCR with a brief reverse transcription (RT) step, hence the designation RT-PCR (Jaykus, 1997). To address these issues, other alternative approaches have been used to simultaneously reduce sample volumes and the levels of interfering compounds. The most frequently applied approach involves isolating and purifying nucleic acids (RNA) from the food sample before RT-PCR (Dubois et al., 2002; Jean et al., 2004; Shan et al., 2005; Guevremont et al., 2006; Dubois et al., 2007; Machado et al., 2009). A second approach

is to capture the virus with a specific antibody. This is more efficient since it specifically isolates the viruses from the different inhibitory substances present in the sample. This is followed by nucleic acid amplification using RT-PCR (Bidawid et al., 2000; Shan et al., 2005; Guevremont et al., 2006).

By combining cell culture and RT-PCR (ICC-RT-PCR), indirect detection of infectivity is obtained by amplification in cell culture followed by an enzymatic amplification (PCR) (Marlowe et al., 2000; Jiang et al., 2004). This method enhances the speed of virus detection in cell culture (Reynolds et al., 1996) and allows for the analysis of larger sample volumes. In addition, dilution of the sample in cell cultures also dilutes any PCR inhibitory substances that may be present in the sample, thus helping to distinguish infectious from non-infectious viruses (Marlowe et al., 2000; Dubois et al., 2002; Jean et al., 2004).

Respiratory Viruses on Produce

2.2 Abstract

Produce consumption has increased in the last two decades, resulting in an increase in the number of foodborne disease outbreaks associated with fresh produce. In addition to foodborne viruses transmitted via fecal contamination, there are emerging zoonotic viral agents such as the respiratory coronavirus, the causative agent of SARS, and influenza virus, which could potentially be transmitted by foods. The goal of this study was to determine the recovery efficiency and the survival of respiratory viruses adenovirus 2 and coronavirus 229E on lettuce, strawberries and raspberries in comparison to the enteric poliovirus 1. Adenovirus was recovered with an efficiency of 56%, 32% and 35% from lettuce, strawberries and raspberries, respectively. Coronavirus was recovered from lettuce with an efficiency of 19.6%, but could not be recovered from strawberries. Poliovirus was recovered from lettuce with an efficiency of 76.6% and 0.06% from strawberries. For comparison purposes, the survival of adenovirus, coronavirus and poliovirus were observed up to eight days on produce. Adenovirus survived the longest on raspberries, with a \log_{10} reduction of 0.61-, followed by 1.68- and 1.75- \log_{10} reductions on strawberries and lettuce, respectively. Coronavirus became inactivated very rapidly; it declined by 0.41- \log_{10} after two days and > 1.34 - \log_{10} by day 4 on lettuce. The enteric poliovirus was more stable than either respiratory virus and survived longer on produce, decreasing by only 0.37- \log_{10} on lettuce and 1.30- \log_{10} on strawberries.

2.3 Introduction

Coinciding with an increase of produce consumption, there has been a significant increase in the number of foodborne disease outbreaks associated with fresh produce in recent years (Rangarajan et al., 2005). Foods may be contaminated by human or animal viruses in two ways: primary contamination due to virus present at the time of harvest, and secondary contamination which occurs during processing, storage, and distribution (Ward et al., 1982). A number of studies suggest that infected food handlers may play an important role in food contamination in many cases (Bidawid et al., 2000). However, proper hygiene measures can reduce the risk of food contamination.

In addition to foodborne viruses transmitted via fecal contamination, there are emerging zoonotic viral agents such as the respiratory coronavirus, the causative agent of SARS, and influenza virus, which could potentially be transmitted by foods, depending on the produce structures (Klein, 2004).

Viruses are incriminated in no less than 69% of acute upper respiratory tract infections (URTI) in the U.S., and are particularly significant as etiologic agents of respiratory infections in the young and the elderly (Klein, 2004). In pediatric respiratory units, adenoviral infections can be devastating; the fatality rate in hospital-acquired cases has been 91%. The hands of attendants are believed to spread the virus (Sattar et al., 2000).

During the SARS outbreak of 2002-2003 in southern China, there was concern that the SARS virus could be spread from one country to another by food (FDA, 2003). Because no previous research has been conducted on the survival of respiratory viruses

on produce, the objective of this study was to assess the recovery efficiency of two respiratory viruses adenovirus 2 and coronavirus 229E on lettuce, strawberries, and raspberries, in comparison to poliovirus 1, an enteric virus. In addition, the survival of the viruses on the different types of produce at 4°C was assessed.

2.4 Materials and methods

Samples. Lettuce (*Lactuca sativa*), strawberries (*Fragaria ananassa*), and raspberries (*Rubus idaeus*) were purchased directly from local produce markets.

Virus and cell lines. Human adenovirus 2 (Ad2) (ATCC-VR846), a respiratory virus, was propagated and assayed in primary liver carcinoma (PLC) (ATCC-8024) cells. Human coronavirus 229E (Co229E) (ATCC-VR740), a respiratory virus, was propagated and assayed on fetal human lung fibroblasts (MRC-5) (ATCC-171) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Poliovirus 1 (PV1) (LSc-2ab) a human enteric virus known to be very stable in the environment, was propagated and assayed on buffalo green monkey kidney (BGMK) cell line. Both were obtained from the Department of Virology and Epidemiology of Baylor College of Medicine, Houston, TX.

Virus propagation. Each of the cell lines were grown to confluent monolayers in 225-cm² flasks and then inoculated with $\sim 10^5$ virus particles. After incubation at 37°C for 30 minutes, 70 ml of Eagle's minimum essential medium (MEM; HyClone Labs. Inc.

Logan, UT) supplemented with 1 mM of sodium pyruvate (NaPyr) was added. Cells were incubated at 37°C and 5% CO₂ until 80% of the monolayer had been destroyed. The flasks were then frozen and thawed three times at -20°C to release the virus. The solution was centrifuged at 1000 x *g* to remove debris; 9% polyethylene glycol (MW 8,000) and 0.5 M sodium chloride were added and solution was stirred overnight at 4°C. After centrifugation at 10,000 x *g* for 30 minutes, the pellet was resuspended in 0.01 M phosphate buffered saline (PBS; pH 7.4; Sigma, St. Louis, MO) to 5% of the original virus suspension volume. Coronavirus was then titered and stored at -80°C. Poliovirus 1 was further purified by extraction with equal volumes of Vertrel XF (Dupont, Wilmington, DE), emulsified and centrifuged at 7,500 x *g* for 15 minutes. Subsequently, the upper aqueous layer containing the viruses was aseptically pipetted into sterile 1-ml vials, titered and stored at -80°C.

Preparation of cell monolayers. Monolayers were trypsinized (Mediatech, Inc., Manassas, VA) and centrifuged for 5 to 10 minutes at 100 x *g* (Dynac, BD, Franklin Lakes, NJ). The cell pellet was resuspended in 10 ml of culture medium with antibiotics, (with 25% antibiotic-antifungal for coronavirus 229E). A cell count was performed using a Neubauer hemacytometer (Hausser Scientific Co., Horsham, PA) and based on cell concentration, the appropriate amount of cell suspension was diluted in the culture medium to give a cell concentration of 1×10^6 cells/ml. Media was agitated by rotation with a magnetic stirrer and the cell suspension was transferred into each of the wells of

cell culture trays. Trays were incubated at 5% CO₂ at 37°C until total confluence of the cells into a monolayer.

Virus assay. Ad2 and CoV229E which do not form plaques in cell culture were assayed by the serial dilution endpoint or Tissue Culture Infectious Dose 50% (TCID₅₀) method, which basically consists on making 10-fold serial dilutions of the stock or samples in pH 7.4 sterile Tris-buffered saline (Sigma Chemical Co., St. Louis, MO). Each dilution was plated on a minimum of six wells into 96-well culture plates. Trays were incubated at 37°C with 5% CO₂ for 30 minutes before addition of the media and subsequent incubation. The cells were then observed for production of cytopathogenic effects (CPE) for 12 days for adenovirus, and 7 days for coronavirus. Titers were determined using the Reed-Muench formula (Payment and Trudel, 1993). Poliovirus 1 was titered in 6-well plastic culture plates by the plaque-forming assay (PFU) method (Payment and Trudel, 1993). Each dilution was plated in duplicate wells.

Virus recovery efficiency. The efficacy of recovery of the different viruses from produce was first assessed. Produce was inoculated with a total of 7.0×10^9 TCID₅₀ adenovirus, 1.2×10^6 TCID₅₀ coronavirus and 1.2×10^8 plaque forming units (PFU) poliovirus. The inoculated produce was then left for 15 minutes at room temperature before processing. All tests were performed in triplicate.

Sample preparation. Lettuce was cut into small pieces approximately 2x2 cm, averaging 8 to 10 g per sample. Two whole pieces of strawberries ranging between 15 to 20 g (for a total of 30 – 40 g per sample), and four-4 to 5 g pieces of raspberries (for a total of 15 to 20 g) were weighed. They were placed in 250-ml autoclavable wide mouth Nalgene™ bottles (VWR, West Chester, PA) with lids, to about one-third capacity.

Sample contamination. In this study, 10 ml of suspensions of poliovirus 1 and adenovirus 2, and 5 ml of coronavirus 229E were inoculated by adding as drops evenly with a pipette onto the produce. Bottles were then covered with the lid and manually rotated to ensure total coverage of the produce by the virus suspension.

Virus extraction. Viruses were eluted from the produce by addition of 90 ml of PBS (pH 9.0) to the bottles for the poliovirus and adenovirus experiments. In order to have a higher initial concentration of virus, only 45 ml of PBS was added for coronavirus (Ward et al., 1982). Produce containing- bottles were shaken for 10 minutes on an orbital shaker (New Brunswick Sci. Co. Inc., Edison, NJ) at 200 RPM to aid in releasing the virus from the produce.

Produce eluates containing organic debris were first clarified by centrifugation at 352 x g for 15 minutes at 5°C (JA-14 rotor, Beckman, Palo Alto, CA) to remove large suspended materials (Shan et al., 2005); however these conditions were not enough to prevent the clogging of membrane filters in the following filtering process, so

centrifugation speed was increased to $1,409 \times g$ for 15 minutes, at 5°C (Butot et al., 2007).

The pH of the eluates was adjusted to pH 7.0-7.4 using 0.1M NaOH or 0.01M HCl. Aliquots of 4 to 7 ml of the neutralized eluates were filtered through a $0.22\text{-}\mu\text{m}$ pore size cellulose nitrate membrane syringe filters (Acrodisc, Pall Co., Ann Arbor, MI) pre-wetted by passing 5 ml of 3% beef extract pH9.0 (Becton Dickinson, Sparks, MD) to prevent virus adsorption to the membrane (Croci et al., 2008). Filtered samples were frozen for subsequent virus assay at -20°C for adenovirus 2 and poliovirus 1 and at -80°C for coronavirus.

Virus survival on produce. To assess survival of the viruses on the produce, contaminated samples were stored in duplicate at 4°C for 14 days for adenovirus, 10 days for poliovirus, and 8 days for coronavirus. After 0, 1, 2, 4, 8, 10, and 14 days, samples were collected, processed, and assayed for virus. All virus survival experiments were performed in duplicate.

Statistic analysis. Student's two-tailed unpaired (assuming unequal variances) t-test was used to compare the survival of viruses on different types of produce. A *P* value of ≤ 0.05 was considered statistically significant.

2.5 Results

2.5.1. Recovery of virus from produce. Table 2.5.1 shows the recovery efficiency of adenovirus 2 from produce. The virus was recovered with 56% efficiency from lettuce. The recoveries for strawberries and raspberries were below 50%, which is the average percent of recovery reported in the literature (Bidawid et al., 2000; Le Guayader et al., 2004; Butot et al., 2007).

Table 2.5.1 Recovery of adenovirus 2 from produce ^{a, b}

Sample	Virus recovered	Average	% Recovery
Lettuce	1.00x10 ⁹ 2.10 x10 ⁹ 1.00 x10 ⁹	1.40 x10 ⁹	56.0
Strawberry	1.00 x10 ⁹ 3.70 x10 ⁸ 1.00 x10 ⁹	8.00 x10 ⁸	32.0
Raspberry	4.00 x10 ⁸ 6.30 x10 ⁸ 1.60 x10 ⁹	8.70 x10 ⁸	35.0

^a Assayed by the TCID₅₀ Method

^b control =2.5x10⁹ (100ml)

The recovery efficiency of human coronavirus 229E was determined only on lettuce and strawberries. Raspberries again were excluded due to the short shelf life of the fruit. Values for recovery were lower, compared to adenovirus 2 and poliovirus 1. Table 2.5.2 shows the recovery of coronavirus from lettuce and strawberries.

Table 2.5.2 Recovery of coronavirus 229E from produce^{a, b}

Sample	Virus recovered	Average	% Recovery
Lettuce	8.88x10 ³ 1.58x10 ⁴ 5.00x10 ³	9.87x10 ³	19.6
Strawberry	ND ^c ND ND		0.0

^a Assayed by the TCID₅₀ Method^b Control= 5.03x10⁴ (25 ml)^c ND- not detected by the method

Table 2.5.3 shows the recovery of poliovirus 1 from lettuce and strawberries. Raspberries were eliminated from the experiments because of their short shelf life before deterioration.

2.5.2 Survival of virus on produce. Table 2.5.4 and Figure 2.5.1 show the adenovirus 2 survival on produce. After 14 days at 4°C the adenovirus titer decreased by 1.11-log₁₀ on raspberries and 2.22- and 2.47-log₁₀ for lettuce and strawberries, respectively. However, after day 8, adenovirus only declined 0.61-log₁₀ on raspberries, and 1.68- and 1.75-log₁₀ on strawberries and lettuce, respectively.

Table 2.5.3 Recovery of poliovirus 1 from produce ^{a, b}

Sample	Virus recovered	Average	% Recovery
Lettuce	1.15x10 ⁷ 1.75x10 ⁷ 1.25x10 ⁷	1.38x10 ⁷	76.70
Strawberry	1.20 x10 ⁴ 9.00 x10 ³ 9.50 x10 ³	1.02x10 ⁴	0.06

^a Assayed by the plaque forming assay method^b Control=1.8x10⁷ (100 ml)Table 2.5.4 Adenovirus 2 survival on produce ^{a, b, c}

Time (days)	Lettuce		Strawberries		Raspberries	
	Virus recovered	Log ₁₀ ^c reduction	Virus recovered	Log ₁₀ ^c reduction	Virus recovered	Log ₁₀ ^c reduction
0	1.44x10 ⁸	0.00	2.10x10 ⁶	0.00	3.61 x10 ⁷	0.00
1	8.68x10 ⁷	0.41	2.50x10 ⁵	1.20**	2.09 x10 ⁷	0.24
2	4.35x10 ⁷	0.61	1.14x10 ⁵	1.29**	2.54 x10 ⁷	0.16
4	3.30x10 ⁷	0.76*	1.00x10 ⁵	1.35**	9.20 x10 ⁶	0.52
8	4.25x10 ⁶	1.75***	1.25 x10 ⁵	1.68	1.19 x10 ⁷	0.61
10	1.88x10 ⁶	1.97***	3.45 x10 ⁴	2.41	3.52 x10 ⁶	1.15
14	1.42x10 ⁶	2.22	2.38 x10 ⁴	2.47	4.78 x10 ⁶	1.11

^a Assayed by the TCID₅₀ Method.^b Average of four replicates from two experiments.^c Average Log₁₀ reduction (-Log₁₀ N_t/N₀ where N_t is titer of virus at specified day and N₀ is titer of virus at time 0).* Statistically different from strawberries ($p \leq 0.05$)** Statistically different from raspberries ($p \leq 0.05$)*** Statistically different from raspberry ($p \leq 0.05$)

Figure 2.5.1. Adenovirus 2 survival on produce

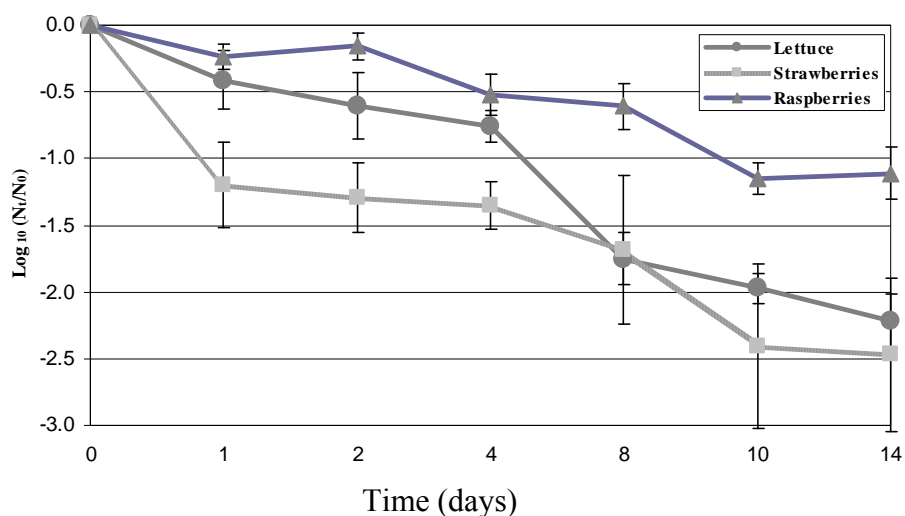


Table 2.5.5 and Figure 2.5.2 show the survival of coronavirus 229 on lettuce.

After 2 days, coronaviruses declined by 0.41-log_{10} and greater than 1.34-log_{10} by day 4.

Table 2.5.5 Coronavirus 229 survival on lettuce^{a, b}

Time (days)	Virus recovered	\log_{10}^d reduction
0	1.02×10^4	0.00
1	2.32×10^4	0.11
2	7.20×10^3	0.41
4	$< 3.94 \times 10^2$ ^c	> 1.34
8	$< 3.94 \times 10^2$ ^c	> 1.34

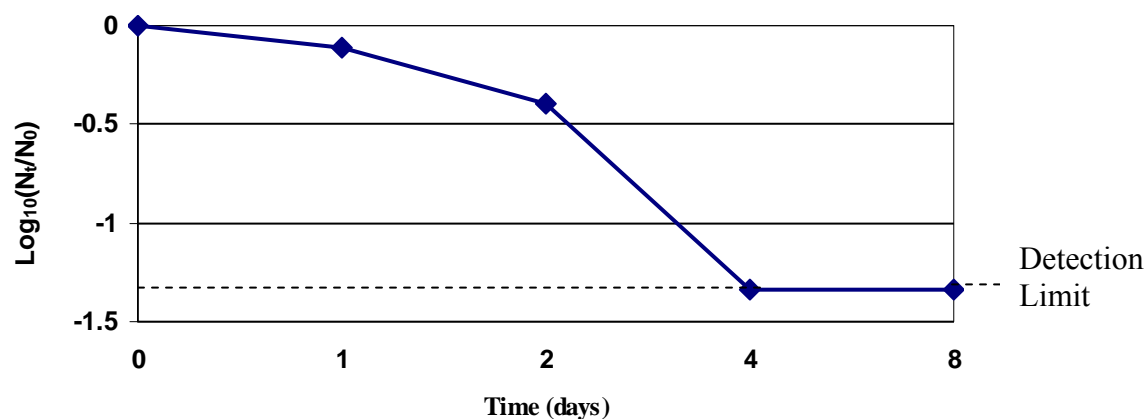
^a Assayed by the TCID₅₀ Method

^b Average of four replicates from two experiments

^c Detection Limit= 3.94×10^2 virus

^d Average \log_{10} reduction ($-\log_{10} N_t/N_0$ where N_t is titer of virus at specified day and N_0 is titer of virus at time 0).

Figure 2.5.2 Coronavirus 229E survival on lettuce



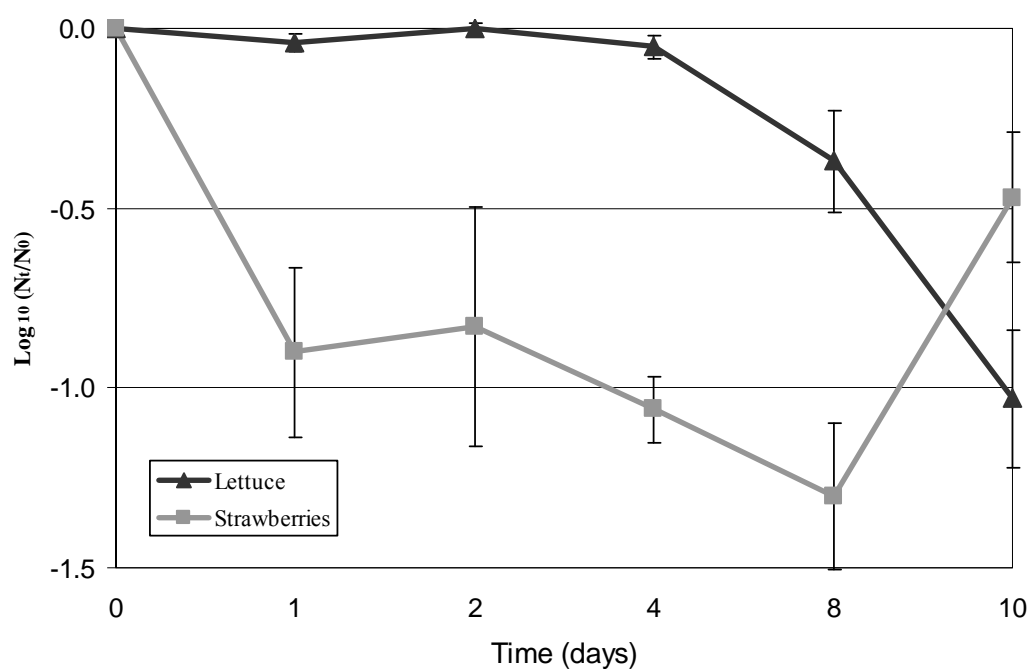
Even though the efficiency of recovery of poliovirus from strawberries (Table 2.5.3) was very low (0.06%), the viral levels recovered could still be used to determine the survival of the virus under refrigeration temperatures (4°C). Poliovirus on lettuce remained fairly stable until day 8 (Table 2.5.6), declining by only 0.37- log_{10} . For strawberries on the other hand, poliovirus remained less stable than on lettuce and the maximum log_{10} reduction reached was 1.30- log_{10} by the same day (Figure 2.5.3).

Table 2.5.6 Poliovirus 1 survival on produce ^{a, b}

Time (days)	Lettuce		Strawberries	
	Virus recovered	Log ₁₀ ^c reduction	Virus recovered	Log ₁₀ ^c reduction
0	9.22x10 ⁶	0.00	6.55x10 ⁴	0.00
1	9.75x10 ⁶	0.04*	1.36x10 ⁴	0.90
2	1.07x10 ⁷	0.00	4.60x10 ⁴	0.83
4	8.52x10 ⁶	0.05*	9.00x10 ³	1.06
8	3.46x10 ⁶	0.37*	6.30x10 ³	1.30
10	9.20x10 ⁵	1.03	2.93x10 ⁴	0.47

^a Assayed by the TCID₅₀ Method.^b Average of six replicates from two experiments.^c Average Log₁₀ reduction ($-\text{Log}_{10} N_t/N_0$ where N_t is titer of virus at specified day and N_0 is titer of virus at time 0).* Statistically different from strawberries ($p \leq 0.05$)

Figure 2.5.3 Poliovirus 1 survival on produce



2.6 Discussion

Many foodborne outbreaks (60%) are caused by viruses, and outbreaks associated with fresh produce have increased over the past decade within the United States (Fino and Kniel, 2008). Even though there have been many studies on the recovery of viruses from fruits and vegetables, there is a lack of standard techniques concerning the initial inoculation and recovery of viruses. This study determined the efficiency in the recovery and survival of poliovirus 1 and two human respiratory viruses, adenovirus 2 and coronavirus 229E on three different types of produce, lettuce, strawberries and raspberries, assayed by cell culture.

The recovery efficiency of 56% for adenovirus in lettuce was comparable to the results obtained by Ward et al. (1982) who recovered an average of 55% of enteric adenovirus type 6 from lettuce. Strawberries and raspberries on the other hand, had recoveries below the percentages reported in other studies. Bidawid et al. (2000) reported a recovery of 81% of Hepatitis A virus (HAV) from strawberries. They used immunomagnetic bead and positively-charged virosorb filters to capture and concentrate HAV. This was followed by its rapid detection by RT-PCR. Virus was quantified by using plaque method.

The differences on the recovery efficiency of adenovirus from strawberries and raspberries in comparison with lettuce on this study may be due to the fact that fruits such as strawberries, raspberries, and blackberries have a porous surface texture which may trap viruses beyond the reach of chemical disinfectants or eluants (Richards, 2001). In addition, the low pH of fruits, which most of the time is below those for vegetables

(strawberry pH is 3.0–3.9, raspberry pH is 2.9–3.5, and iceberg lettuce pH is approximately 6.03) (Martin et al. 2005; ICMSF, 2005a; ICMSF, 2005b) may affect the sorption of the virus to the produce surface and therefore, their extraction.

Adenoviruses are usually very stable to chemical and physical agents and adverse pH conditions due to the double stranded DNA genome which, if damaged, may be repaired by host cell DNA repair mechanisms; however, a basic buffer (pH 7.4 to 9.5) is recommended to extract the viruses because acidic fruits such as raspberries can reduce the pH of the eluant during the elution process, which may lead to the pH dropping below neutrality, reducing virus elution (Crocì et al., 2008). In addition, some fruits, including strawberries, blueberries, and grapes contain quercetin, a close chemical relative of resveratrol, which has been shown to have anti-viral properties in cell culture experiments (Davis et al., 2008).

Coronavirus recovery efficiency from lettuce was only 19.6%. The virus inoculated on strawberries was not detected by this method. The fruit acidity may make them less stable in the environment than non-enveloped viruses. Shan et al. (2005) could detect 100 pfu/ml of HAV from seeded strawberries rinses, but only one-third of samples seeded with 10 pfu/ml of HAV were positive.

Poliovirus was recovered with an efficiency of 76.7% from lettuce, which was greater than the 58% of poliovirus 1 recovered from lettuce by Ward et al. (1982). Recovery of poliovirus 1 on lettuce was also greater than the recovery of adenovirus 2 on the same produce in this study. This may be because human enteric viruses such as

poliovirus 1 can withstand a wide range of pH and temperature variations (Jaykus, 2000). Recovery of poliovirus 1 from strawberries was 0.06%.

Butot et al. (2007) found recovery efficiencies of 1.99, 0.42, and 1.1% of Hepatitis A (HAV), norovirus (NV), and rotavirus (RV), respectively from fresh strawberries, and 2.3, 3.07, and 1.3%, for HAV, NV, and RV, respectively from frozen raspberries. This low efficiency may be due to the rougher surface of strawberries or to greater damage to the surface of the fruit during processing resulting in greater retention of the virus. This may also be due to the natural occurrence of acidic pH and inhibitors (flavonoids such as quercetin) in the fruits.

Produce were stored in lid-covered bottles to avoid evaporation of the inoculum during storage; however, fruits started to deteriorate before day 10 due to moisture excess. During postharvest transportation, produce shelf life can be improved by using controlled atmospheres while keeping the transport temperatures between 0°C and 5°C, increasing the transit/postharvest life of strawberries up to 5 to 10 days (TransFresh, 2004); however strawberry producers recommend not to wash berries until just before serving, and to avoid contact with moisture, misters, etc. in the food market. Otherwise, moisture will cause rapid breakdown of the berries (Pacific Gold Farms, 2003b). They state that under ideal conditions, strawberries should have a shelf life of 2 to 5 days in a consumer's refrigerator.

After harvest, even the most premium berries, as a normal process, will begin to degrade and deteriorate, losing freshness and nutrients before reaching store displays. Raspberries are very perishable and should always be refrigerated between 0 and 1°C.

They are very sensitive to temperatures over 1°C, and under ideal conditions, raspberries should have a shelf life of 1 to 2 days in the consumer's refrigerator (Pacific Gold Farms, 2003a). This was confirmed during this study since raspberries started to deteriorate faster than strawberries, and for this reason, the survival of poliovirus and coronavirus on raspberries was not determined.

After day 8, during the adenovirus survival experiments, strawberries were swollen, raspberries started to show black fungal growth, and lettuce started to get brown edges due to enzymatic reactions. After 10 days, the berries appeared dry, with some condensation inside the bottle walls. The strawberries and raspberries were partially covered by fungi and had off odors. After day 14, soft fruits were totally covered by fungi and exudates from those samples. For these reasons and for comparison purposes, survival of adenovirus, coronavirus and poliovirus were observed only up to day eight.

Adenovirus 2 survived the longest on raspberries, followed by strawberries, and then by lettuce. Differences in adenovirus decaying between raspberries and strawberries may be due to uneven virus distribution over the fruit surfaces; however the differences were not significant. Differences in \log_{10} reductions between raspberries and lettuce were significant after days 8 and 10, and again, may be due to the porous surface texture which may trap viruses beyond the reach of chemical disinfectants or eluants (Richards, 2001). In addition, the low pH and inhibitors in the fruits may also have an effect. Le Guayader et al. (2004) reported that detecting viruses in berries, especially raspberries, is a difficult task due to the presence of various inhibitors and a low pH.

Coronavirus 229E was very sensitive to the extraction process. After 2 days, coronaviruses declined by 0.41-log_{10} and $>1.34\text{-log}_{10}$ on lettuce after only four days. These results are comparable with those obtained by Gundy et al. (2009) who reported that coronaviruses die-off very rapidly in wastewater.

The enteric poliovirus 1 was more stable than either respiratory virus and survived longer on produce. These differences were significant. Survival studies of poliovirus on soft fruit and salad vegetables done by Kurdziel et al. (2001) reported that there was a significant linear decline of poliovirus of 0.086-log_{10} per day for lettuce stored at 4°C for 15 days. There was a decline of 0.12-log_{10} of poliovirus on frozen strawberries stored at -20°C for 15 days. Raspberry samples displayed severe deterioration by day 9, but no significant decline in poliovirus was observed. Croci et al. (2002) found that lettuce had more HAV adsorption capacity on lettuce with only a slight decrease of 0.004-log_{10} reduction observed after day 9 at 4°C .

Flat surfaces allow for greater evaporation of the suspending inoculum than the more complex surfaces provided by raspberries and strawberries. Some viruses (coxsackie B5 virus, echovirus, and reovirus) can survive up to 6 days on strawberries under humid conditions without any reduction in inoculated numbers; however, if the fruit sample is allowed to dry, only 1% of the inoculum could be recovered. Desiccation has a negative effect on enteric virus survival on surfaces and therefore on virus persistence on foodstuffs. Poliovirus is less resistant to desiccation than HAV or rotavirus (RV) (Kurdziel et al., 2001).

Unhygienic practices by food handlers may result in the transmission of viral agents such as respiratory adenovirus and coronavirus that infect mucosal membranes and the respiratory tract. The results of this study show that respiratory viruses may persist on fresh fruit and vegetables for several days under conditions commonly used for storage in households. Considering the low infectious dose of viral agents there will always be a risk of infection from consumption of the food if no regular preventive produce surface disinfection is performed.

CHAPTER 3

QUANTITATIVE MICROBIAL RISK ASSESSMENT

3.1 Microbial risk assessment

Pathogen risk assessment is a process that evaluates the likelihood of adverse human health effects following exposure to a pathogenic organism (RAWG, 1996). Quantitative microbial risk assessment (QMRA) follows the same general paradigm for chemical risk assessment which consists of four steps: hazard identification, exposure assessment, dose-response assessment, and risk characterization (Nwachuku and Gerba, 2004).

Hazard identification defines the hazard and nature of the adverse effect. In the case of pathogens, this is complicated because of the differences in severity of the outcomes (from asymptomatic infection to development of clinical illness to death), and secondary or person to person transmission (the attack rate). The severity of the outcomes in turn, depend on the characteristics of the host (preexisting immunity, age, nutrition, and ability to mount an immune response, etc.) and the nature of the pathogen, with factors such as type and strain of the organism and its capacity to elicit an immune response (Gerba, 2000).

Exposure assessment determines the intensity, frequency, and duration of human exposure to an environmental agent. Many elements may be included in the analysis,

such as the vehicle food or water, the associated unit of exposure (number of organisms ingested), the size and demographics of the potentially exposed population, the route of exposure, and transfer rates. Another critical consideration for pathogen risk assessment is the potential for changes in pathogen concentration due to growth or death, or the fact that pathogenic microorganisms may be unevenly distributed in water or other media due to clumping or aggregation (RAWG, 1996). For intake or exposure quantification, models are employed which involve three sets of variables: the concentration of microbes in the media, the exposure rates which relate the magnitude with the frequency or duration of exposure, and the quantified biological characteristics of receptors such as body weight, level of immunity to microbial pathogens, etc. Most exposure concentrations are derived from measured and/or model data (Gerba, 2000).

Dose-response assessment evaluates the relationship between dose, infectivity, and the manifestation of clinical illness (response). Some toxicity data are derived from occupational, clinical, and epidemiological studies, but most toxicity data come from animal experiments. Data from dose-response curves derived from animal studies are obtained by examining the effects of large doses on test animals. Low-response doses must be extrapolated from the high -dose data. To resolve the controversy surrounding this issue, several mathematical models have been proposed to extrapolate from high to low doses (Gerba, 2000). The analysis is affected by the quality and quantity of data available for the assessment of human health effects; sometimes knowledge of the actual dose may be limited (RAWG, 1996).

Risk characterization step combines all the information learned from the first three steps and, in a quantitative risk assessment, computes a numerical estimate of risk which reflects the likelihood that a microorganism will cause an adverse health effect within a population, and the severity of the effect (Mena et al., 2004).

3.1.1 Quantitative microbial risk assessment of respiratory adenovirus 2 on lettuce.

Hazard identification. Human adenovirus (Ads) is member of the family Adenoviridae and the genus *Mastadenovirus* (mammal). The 51 serotypes of Ads have been classified into six species, from A to F, according to their biological properties (van Heerden et al., 2005). Although many adenovirus infections are asymptomatic, these viruses can cause acute respiratory disease (ARD) (types 1-7, 14, and 21), conjunctivitis (types 3, 7, 8, 11, 14, 19, and 37), acute hemorrhagic cystitis (types 11 and 21), ARD of military recruits (types 3, 4, 7, 14, and 21), and gastroenteritis (types 31, 40 and 41) (Rusin et al., 2000). About 5% of respiratory diseases in children under 5 years old and 3% in adults is due to adenovirus infections. The disease is usually mild, but types 4 and 7 have caused epidemics of ARD in up to 80% of recruits in a military unit, 20 to 40% of which have required hospitalization (Strauss and Strauss, 2002).

Exposure assessment. Certain respiratory infections are transmitted by direct and indirect contact, while others are transmitted through exposure to contaminated large droplets emitted during coughing and sneezing. Viral shedding has been detected in nasal secretions, for example, with up to 10^7 infectious influenza viral particles per ml (Boone

and Gerba, 2005). Research performed in 1900 by Koeniger regarding bacterial dissemination by droplets from the mouth, during coughing and sneezing showed that the greatest distance to which bacteria were carried in the experiments was 12.40 meters (Reiling, 2000). Airborne viruses present in sneezing can travel at speeds of around 150 km/hour. This is therefore a very efficient way of spreading germs (Mullins, 2003) and contamination of lettuce in the field by infected food handlers is also possible.

For any environmental contamination to be relevant, the virus should not only remain infectious but also persist at a sufficient concentration to enable it to reach the respiratory tract via finger contamination (Thomas et al., 2008). Adenovirus has a double-stranded DNA genome which renders them more resistant to UV-light than enteroviruses. This is due to the repair of UV-induced pyrimidine dimers in the viral genome by host cell DNA mechanisms for which both Ads DNA strands may serve as a template for replication if one strand is damaged by environmental factors (van Heerden et al., 2005).

The amount of lettuce consumed per person in the United States was determined by using the annual per capita consumption of lettuce, estimated at 4,416.5 g. (USDA, 1995)

Dose-response assessment. There are no previous studies with data concerning the natural occurrence of adenovirus 2 on lettuce; however, quantitative studies have indicated that the number of viral particles necessary to produce infection in the

respiratory tract is relatively small. With Adenovirus, for example, this number is on the order of seven virions (Kaslow and Evans, 1997).

Risk characterization. The objective of this study was to assess the risks (P_i), associated from ingesting 1, 10, and 100 infectious particles of Adenovirus 2, during one serving and ten servings of lettuce, and 365 servings a year. Based on data obtained from human-dose response studies, the following exponential model was chosen:

$$P_i = 1 - \exp(-rN)$$

Where:

P_i represents the probability of becoming infected

N represents the number of microorganisms ingested or inhaled

r is a constant parameter = 0.4172 from a dose-response study with adenovirus type 4 (Crabtree et al., 1997).

The probability of becoming infected after ingestion of ten servings was calculated by using:

$P_{10} = 1 - (1 - p_i)^{10}$ and the yearly risk was calculated using:

$P_{\text{year}} = 1 - (1 - p_i)^{365}$ (Gerba, 2000).

Table 3.1.1 shows the risk of infection associated with the ingestion of lettuce contaminated with Adenovirus 2.

Table 3.1.1 Risk associated with ingestion of human adenovirus 2^a

No. of viruses per serving [*]	No. of servings of lettuce		
	One	Ten	365
1	3.4×10^{-1}	9.8×10^{-1}	1.0
10	9.8×10^{-1}	1.0	1.0
100	1.0	1.0	1.0

^a by using the exponential model

^{*} assuming 12.1 g of lettuce per serving (based on per capita consumption per year)

Assuming that 100% of the virus particles on the lettuce are transferred to the hands and then to the nose, the estimated risk of infection from one particle of adenovirus 2 in one serving of lettuce (assuming 12.1 g of lettuce per serving), is 3.4×10^{-1} . Risk of infection increased to 9.8×10^{-1} when exposure was increased to ten viral particles per serving, as well as by increasing servings to ten. The estimated risk of ingestion of ten virions in ten servings was 1.0, as well as by increasing the viral particles to 100. The risk associated to the ingestion of one, ten or one hundred virion particles was always 1.0 when consumed in 365 servings.

3.1.2 Assessment of risk of infection with Ad2 by an infected food handler during harvesting of lettuce.

Since not all the virus particles on the lettuce will be transferred to the hands or the nose, the transfer rates for each step need to be taken into consideration to more accurately determine the risk. Rusin et al. (2002) carried out a study to determine the transfer efficiency of certain bacteria, and coliphage PRD-1 as a surrogate for viruses, from surfaces to hand and from hand to mouth. They found that the transference of PRD-1 from carrot to the hand was 0.35%, and from hands to lips was 33.9%. Taking these values into consideration, the risks from Table 3.1.1 were adjusted, assuming the transfer of Adenovirus from lettuce to hands and from hands to nose. N values for 1, 10 or 100 infectious particles therefore become 0.0012, 0.012, and 0.12, respectively.

Table 3.1.2 shows the risk of infection associated with the transfer of adenovirus 2 from lettuce to hand and from hand to nose. The estimated risk of infection from one adenovirus by ingesting one serving of lettuce (assuming ingestion of 12.1 g of lettuce per serving) was 5.0×10^{-4} or almost 1 in 2,000. The risk of infection increased to 5.0×10^{-3} (1 in 200) on ten servings, and to 1.7×10^{-1} (1 in 6) on 365 servings.

The estimated risk of infection from the ingestion of ten viruses of adenovirus in one single serving was 5.0×10^{-3} (1 in 200), the same as for ingestion of ten servings of one single particle). The risk increased to 4.9×10^{-2} (1 in 20) by the ingestion of ten servings, and to 8.4×10^{-1} (1 in 2) by ingesting 365 servings. Likewise the estimated risk of infection from the ingestion of one hundred viruses in one serving was 4.9×10^{-2} (1 in

20) and increased to 4.0×10^{-1} (1 in 2.5) by increasing the servings to ten. The risk of infection increased to 1.0 (1 in 1) on 365 servings.

Table 3.1.2 Risk associated with transfer^a of human adenovirus 2 from lettuce to the nose.

No. Virus Per serving	No. of servings of lettuce		
	One	Ten	365
1	1/2000	1/200	1/6
10	1/200	1/20	1/2
100	1/20	1/2.5	1/1

^a assuming 0.35% transfer from produce to hands and 33.9% from hands to nose.

On the other hand, considering the number of viral particles necessary to produce infection in the respiratory tract (Kaslow and Evans, 1997), and the same transfer efficiencies as in the Rusin et al. study (2002), the estimated risk of inhaling seven virions from adenovirus contaminated lettuce, was 3.9×10^{-3} in one single serving of lettuce, meaning that approximately 0.4 % of people would be infected at this dose.

Even though the consumption of respiratory viruses on produce does not represent a risk of enteric infection, it represents the possibility of transmission of the virus from produce to hands and from hands to the mouth/nose, possibly causing respiratory infections. Respiratory viruses are able to survive on produce for periods of time

sufficient to reach the consumer and depending on the initial level of contamination, they could represent a health risk to consumers.

From this assessment it appears that respiratory viruses that are able to survive on produce during storage could be transmitted to consumers. Studies with coronaviruses suggest that their survival is shorter in duration and their removal from produce surfaces are lower than those observed with adenoviruses. Thus, the likelihood of coronaviruses on produce to cause respiratory infections in consumers may be far less than that predicted for adenoviruses.

APPENDIX A

IDENTITY AND NUMBERS OF BACTERIA PRESENT IN TABLETOPS
AND IN DISHCLOTHS USED TO WIPE DOWN TABLETOPS IN PUBLIC
RESTAURANTS AND BARS¹

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Department of Soil, Water and Environmental Science
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Running Title: Bacterial Occurrence on Tabletops/Dishcloths in Restaurants.

A peer-reviewed article

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APPENDIX B

VIRAL SURVIVAL DATA FROM FRESH PRODUCE

Table 1. Survival of adenovirus 2 on produce^a

Day	Lettuce				Strawberries				Raspberries			
	Virus recovered ^c	Average	Log ₁₀ N _t /N ₀ [*]	Log ₁₀ ^b reduction	Virus recovered ^c	Average	Log ₁₀ N _t /N ₀ [*]	Log ₁₀ ^b reduction	Virus recovered ^c	Average	Log ₁₀ N _t /N ₀ [*]	Log ₁₀ ^b reduction
0	1.12x10 ⁸				2.00x10 ⁶				5.02 x10 ⁷			
	6.32 x10 ⁷	8.76 x10 ⁷	0.00		7.96 x10 ⁵	1.40 x10 ⁶	0.00		6.32 x10 ⁷	5.67 x10 ⁷	0.00	
	2.00 x10 ⁸				2.00 x10 ⁶				1.12 x10 ⁷			
	2.00 x10 ⁸	2.00 x10 ⁸	0.00	0.00	3.56 x10 ⁶	2.80 x10 ⁶	0.00	0.00	2.00 x10 ⁷	1.56 x10 ⁷	0.00	0.00
1	2.00 x10 ⁸		-0.35		7.96 x10 ⁵		0.25		3.56x10 ⁷		0.20	
	6.32 x10 ⁷	1.32 x10 ⁸	0.14		6.32 x10 ⁴	4.30 x10 ⁵	1.35		2.00 x10 ⁷	2.78 x10 ⁷	0.45	
	6.32 x10 ⁷		0.50		7.96 x10 ⁴		1.55		2.00 x10 ⁷		-0.11	
	2.00 x10 ⁷	4.16 x10 ⁷	1.00	0.41	6.32 x10 ⁴	7.14 x10 ⁴	1.65	1.20	7.96 x10 ⁶	1.40 x10 ⁷	0.30	0.24
2	2.00 x10 ⁷		0.64		2.00 x10 ³		0.85		2.00 x10 ⁷		0.45	
	7.96 x10 ⁷	4.98 x10 ⁷	0.04		1.12 x10 ³	1.56 x10 ³	1.10		5.02 x10 ⁷	3.51 x10 ⁷	0.05	
	1.12 x10 ⁷		1.25		7.96 x10 ²		1.55		2.00 x10 ⁷		-0.11	
	6.32 x10 ⁷	3.72 x10 ⁷	0.50	0.61	6.32 x10 ²	7.14 x10 ²	1.65	1.29	1.12 x10 ⁷	1.56 x10 ⁷	0.14	0.16
4	9.28 x10 ⁶		0.97		2.00 x10 ⁵		0.85		1.12 x10 ⁷		0.70	
	9.28 x10 ⁶	9.28 x10 ⁶	0.97		6.32 x10 ⁴	1.32 x10 ⁵	1.35		7.96 x10 ⁶	9.60 x10 ⁶	0.85	
	5.02 x10 ⁷		0.60		7.54 x10 ⁴		1.57		6.32 x10 ⁶		0.40	
	6.32 x10 ⁷	5.67 x10 ⁷	0.50	0.76	6.32 x10 ⁴	6.93 x10 ⁴	1.65	1.35	1.12 x10 ⁷	8.80 x10 ⁶	0.14	0.52
8	7.96 x10 ⁵		2.04		3.54 x10 ⁵		0.60		2.00 x10 ⁷		0.45	
	6.32 x10 ⁵	7.14 x10 ⁵	2.14		1.12 x10 ⁵	2.32 x10 ⁵	1.10		2.00 x10 ⁷	2.00 x10 ⁷	0.45	
	6.32 x10 ⁶		1.50		3.56 x10 ⁴		1.90		6.32 x10 ⁶		0.40	
	9.28 x10 ⁶	7.80 x10 ⁶	1.33	1.75	2.00x10 ³	1.88 x10 ⁴	3.15	1.68	1.12 x10 ⁶	3.72 x10 ⁶	1.14	0.61
10	9.28 x10 ⁵		1.97		4.30 x10 ⁴		1.51		6.32 x10 ⁶		0.95	
	5.02 x10 ⁵	7.14x10 ⁵	2.24		9.30 x10 ⁴	6.80 x10 ⁴	1.18		6.32 x10 ⁶	6.32 x10 ⁶	0.95	
	4.10 x10 ⁶		1.69		<7.96x10 ^{2d}		>3.55		6.32 x10 ⁵		1.40	
	2.00 x10 ⁶	3.05 x10 ⁶	2.00	1.97	1.12x10 ³	9.58x10 ²	3.40	2.41	7.96 x10 ⁵	7.14 x10 ⁵	1.30	1.15
14	6.32 x10 ⁵		2.14		5.02 x10 ⁴		1.45		1.12 x10 ⁷		0.70	
	2.00 x10 ⁵	4.16 x10 ⁵	2.64		4.30 x10 ⁴	4.66 x10 ⁴	1.51		6.32 x10 ⁶	8.80 x10 ⁶	0.95	
	4.10 x10 ⁶		1.69		1.12x10 ³		3.40		3.56 x10 ⁵		1.64	
	7.54 x10 ⁵	2.43 x10 ⁶	2.42	2.22	<7.96x10 ^{2d}	9.58x10 ²	>3.55	2.47	1.12 x10 ⁶	7.40 x10 ⁵	1.14	1.11

^a Assayed by the TCID₅₀ Method.^b Average - Log₁₀ reduction used for the graphics.^c Based on 100 ml extraction^d Detection Limits *Negative values were taken as zero and considered on averages

Table 2. Survival of coronavirus 229E on produce^a

Day	Lettuce			
	Virus recovered	Average	Log ₁₀ N _t /N ₀ *	Log ₁₀ ^b reduction
0	9.00x10 ³	1.58 x10 ⁴	0.00	0.00
	2.25 x10 ⁴		0.00	
	3.91 x10 ³	4.70x10 ³	0.00	
	5.50 x10 ³		0.00	
1	5.60x10 ³	1.54 x10 ⁴	0.45	0.11
	2.51 x10 ⁴		-0.20	
	3.10 x10 ⁴	3.10 x10 ⁴	-0.82	
	3.10 x10 ⁴		-0.82	
2	1.78 x10 ³	2.88x10 ³	0.95	0.40
	3.98 x10 ³		0.60	
	1.74 x10 ⁴	1.15x10 ⁴	-0.57	
	5.50 x10 ³		- 0.07	
4	<3.98x10 ²	<3.98x10 ²	>1.60	>1.34
	<3.98x10 ²		>1.60	
	<3.91x10 ²	<3.91x10 ²	>1.08	
	<3.91x10 ²		>1.08	
8	<3.98x10 ²	<3.98x10 ²	>1.60	>1.34
	<3.98x10 ²		>1.60	
	<3.91x10 ²	<3.91x10 ²	>1.08	
	<3.91x10 ²		>1.08	

^a Assayed by the TCID₅₀ Method^b Average-Log₁₀ Reduction used for the graphics^c Detection Limit 3.98x10² 1st. exp. (50 ml extraction volume)^d Detection Limit 3.91x10² 2nd. exp. (49.1 ml extraction volume)

* Negative values were taken as zero and considered on averages

Table 3. Survival of poliovirus 1 on produce^a

Time (days)	Lettuce				Strawberries			
	Virus recovered	Average	Log ₁₀ N _t /N ₀ [*]	Log ₁₀ ^b	Virus recovered	Average	Log ₁₀ N _t /N ₀ [*]	Log ₁₀ ^b
0	3.65x10 ⁶				7.50x10 ⁴			
	3.65 x10 ⁶				1.10 x10 ⁴			
	3.65 x10 ⁶	3.65 x10 ⁶	0.00		3.50 x10 ⁴	4.03 x10 ⁴	0.00	
	1.30 x10 ⁷				9.50 x10 ⁴			
	1.55 x10 ⁷				8.75 x10 ⁴			
	1.60 x10 ⁷	1.48 x10 ⁷	0.00	0.00	9.00 x10 ⁴	9.08 x10 ⁴	0.00	0.00
1	2.80 x10 ⁶		0.12		4.05 x10 ⁴		-0.002	
	6.15 x10 ⁶		-0.23		7.50 x10 ³		0.73	
	6.05 x10 ⁶	5.00 x10 ⁶	-0.22		<5.00x10 ^{2c}	2.43x10 ⁴	>1.90	
	1.10 x10 ⁷		0.13		6.25 x10 ³		1.16	
	1.45 x10 ⁷		0.009		5.25 x10 ³		1.24	
	1.80 x10 ⁷	1.45 x10 ⁷	-0.09	0.04	4.00 x10 ⁴	2.29 x10 ⁴	0.36	0.90
2	5.05 x10 ⁶		-0.14		2.30 x10 ⁴		0.24	
	4.55 x10 ⁶		-0.09		1.00 x10 ³		1.60	
	5.45 x10 ⁶	5.01 x10 ⁶	-0.17		<5.00x10 ^{2c}	8.20x10 ³	>1.90	
	1.65 x10 ⁷		-0.05		1.27 x10 ⁵		-0.14	
	1.80 x10 ⁷		-0.09		1.97 x10 ⁵		-0.34	
	1.50 x10 ⁷	1.65 x10 ⁷	-0.006	0.00	5.50 x10 ³	8.38 x10 ⁴	1.22	0.83
4	5.65 x10 ⁶		-0.19		2.50 x10 ³		1.20	
	5.30 x10 ⁶		-0.16		4.50 x10 ³		0.95	
	5.40 x10 ⁶	5.45 x10 ⁶	-0.17		<5.00x10 ^{2c}	2.50x10 ³	>1.90	
	1.35 x10 ⁷		0.04		1.60 x10 ⁴		0.75	
	9.50 x10 ⁶		0.20		1.92 x10 ⁴		0.67	
	1.20 x10 ⁷	1.16 x10 ⁷	0.09	0.05	1.13 x10 ⁴	1.55 x10 ⁴	0.90	1.06
8	3.65 x10 ⁶		0.00		5.00 x10 ²		1.90	
	3.10 x10 ⁶		0.07		3.50 x10 ³		1.06	
	2.10 x10 ⁶	2.95 x10 ⁶	0.24		<5.00x10 ^{2c}	1.50x10 ³	>1.90	
	6.50 x10 ⁶		0.36		4.50 x10 ³		1.30	
	3.65 x10 ⁶		0.61		1.35 x10 ⁴		0.83	
	1.80 x10 ⁶	3.98 x10 ⁶	0.91	0.37	1.55 x10 ⁴	1.11 x10 ⁴	0.77	1.30
10	1.15 x10 ⁶		0.50		5.25 x10 ⁴		-0.11	
	3.70 x10 ⁵		0.99		2.80 x10 ⁴		0.16	
	2.35 x10 ⁵	2.40 x10 ⁵	1.19		1.10 x10 ⁴	3.05 x10 ⁴	0.56	
	1.45 x10 ⁶		1.00		1.07 x10 ⁴		0.93	
	3.15 x10 ⁶		0.67		8.25 x10 ³		1.04	
	2.20 x10 ⁵	1.60 x10 ⁶	1.83	1.03	6.50 x10 ⁴	2.80 x10 ⁴	0.15	0.47

^a Assayed by the plaque forming unit method^b Average Log₁₀ used for the graphics^c Detection Limits

*Negative values were taken as zero and considered on averages

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