

ABSTRACT

Title of Thesis: DETECTION OF NOROVIRUS AND
INDICATOR ORGANISMS ON FRESH
PRODUCE.

Karen E. Williams, Master of Science, 2005

Directed By: Jianghai Meng, Associate Professor,
Department of Nutrition and Food Science.

Food borne illness is a major problem around the world. Recently, more food borne outbreaks involve produce as the vehicle and viruses as the source of contamination. Norovirus is a common food borne viral pathogen. Genetic diversity among the viruses has made detection difficult. Due to the difficulties in detection, the norovirus is an ideal candidate for having an indicator organism. FRNA bacteriophages share several similarities with enteric viruses and would be an ideal candidate. In this study, we evaluated reverse transcriptase polymerase chain reaction (RT-PCR) detection of norovirus and evaluated using FRNA bacteriophages, *E. coli*, and *Enterococcus* as indicator organisms for the virus on produce. Of the five RT-PCR methods tested, only two worked with both controls. Of the 180 produce samples tested, 37.2% were positive for FRNA bacteriophage, 17.2% were positive for *Enterococcus*, and 0% were positive for *E. coli*. We conclude that RT-PCR is not an efficient method for screening norovirus on produce and including FRNA bacteriophages as indicator organisms for viruses may help decrease outbreaks.

DETECTION OF NOROVIRUS AND INDICATOR ORGANISMS ON FRESH
PRODUCE

By

Karen Elizabeth Williams

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2005

Advisory Committee:
Professor Jianghong Meng, Chair
Professor Martin Lo
Professor Mark Kantor

© Copyright by
Karen Elizabeth Williams
2005

Table of Contents

Table of Contents	ii
List of Tables	iii
List of Figures	iv
Introduction.....	1
Produce related food safety.....	4
Food borne viruses.....	7
Indicator organisms.....	12
Goals of study	15
Materials and Methods.....	16
Results.....	21
Discussion.....	23
Conclusions.....	27
References.....	37

List of Tables

1. Primer sequences used in RT-PCR – p. 28
2. Prevalence of FRNA bacteriophage, *E. coli*, and *Enterococcus* on fresh produce samples – p.29

List of Figures

1. Spot enrichment assay methods – p. 30
2. Protocols for *E. coli* detection – p.31
3. RT-PCR results for MON primer set – p.32
4. RT-PCR results for NV primer set – p. 33
5. RT-PCR results for SR primer set – p.34
6. Spot agar dish – p. 35
7. FRNA bacteriophage prevalence by store – p.36

Introduction

There are an estimated 76 million cases of food borne illness each year leading to 325,000 hospitalizations and 5,200 deaths per year (52). There are over 200 known diseases that can be transmitted through food (9). Diseases caused by food borne pathogens can be as mild as a slight case of diarrhea to as severe as death. *Salmonella*, *Listeria*, and the parasite *Toxoplasma* are responsible for 80% of estimated food-related deaths (52).

New technologies and food processing techniques have been developed to eliminate or reduce food borne disease. For example, proper thermal canning of food greatly decreases illnesses caused by *Clostridium botulinum* (25) and pasteurization has made milk and dairy products safer. However, in the last 20 years new pathogens have emerged and become a significant cause of disease. *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Cyclospora cayetanensis* are all pathogens that were not recognized as food borne pathogens years ago. Despite technological advances, food borne disease is still a major problem.

FoodNet is a surveillance program run by the Centers for Disease Control (CDC) that quantifies the incidence of food borne disease through surveillance of laboratory-diagnosed illnesses (30). The preliminary FoodNet data from 2004 reports *Salmonella*, *Campylobacter*, *Shigella*, *Cryptosporidium*, and *Escherichia coli* O157:H7 as the leading bacterial and parasitic causes of food borne disease (14). In addition, *C. botulinum*, *L. monocytogenes*, norovirus, *Toxoplasma gondii*, *Vibrio vulnificus*, and *Staphylococcus aureus* are also problematic food borne pathogens (23). *Salmonella* is the most common cause of death from food borne disease; sources

include raw or undercooked eggs. *Campylobacter* is the most common cause of bacterial diarrhea in the U.S.; sources are most often raw or undercooked meat and poultry. *Shigella* is often found in salads, milk, and other dairy products. *Cryptosporidium* is a parasite that causes severe diarrheal disease. *E. coli* O157:H7 causes severe gastroenteritis that can lead to Hemolytic Uremic Syndrome (HUS). Sources of *E. coli* O157:H7 include, but are not limited to, undercooked ground beef and fresh produce. *C. botulinum* produces a deadly toxin and its' source is often home-prepared foods. *L. monocytogenes* is especially dangerous to pregnant women and newborns and is often found in ready-to-eat foods such as unpasteurized dairy products and meat products. Norovirus is the leading cause of diarrhea in the US; sources include shellfish and produce. *T. gondii* is a parasite found in raw or undercooked meats. *V. vulnificus* causes gastroenteritis and is usually found in raw or undercooked seafood. Finally, *Staphylococcus aureus* produces a heat resistant toxin that causes vomiting shortly after consumption of the contaminated food. *S. aureus* can be present in a wide variety of foods.

In order to better control food borne disease the federal government requires food manufacturers and distributors to develop Hazard Analysis and Critical Control Points (HACCP) programs (55). HACCP helps manufacturers and food processors identify points in their processing method at which contamination could occur and how to prevent it from occurring. The main points of HACCP include: to develop a HACCP plan to identify and control pathogens in their products, to meet targets for the reduction of microbial pathogens, to conduct microbial testing to determine compliance with targets, and finally to establish and follow written standard operating

procedures. HACCP procedures are in effect for almost all areas of the food industry including meat, poultry, seafood, and produce.

HACCP was mandated in 1996 for large food manufacturers and was gradually introduced in to the entire food industry (62). Studies have been done to show the impact of HACCP. In one study (62), raw meat and poultry samples were taken from federally inspected slaughterhouses and tested for the prevalence of *Salmonella*. Samples were taken from 1998 through 2000, the first few years that HACCP was introduced. In most cases the prevalence of *Salmonella* was lower than it was prior to HACPP, however *Salmonella* was still present. This demonstrates that although laws are being established to help decrease food borne pathogens, the pathogens still persist.

Food borne disease has been a problem for a long time and will likely continue to be a problem in the future. However, with the introduction of new laws, such as HACCP, and the development of new detection methods, the incidence of disease may decrease. In addition, as new pathogens emerge and as foods associated with disease change, detection methods for pathogens may need to be revised or adapted to fit new needs.

Produce related food safety

An increasing amount of fresh fruits and vegetables are associated with food borne illnesses each year. In a study by Sivapalasingham *et al* that summarized outbreak data from 1973 to 1997 (64), there were 190 outbreaks of food borne disease associated with fresh produce. As a result of these outbreaks, there were 16,058 reported illnesses, 598 hospitalizations, and 8 deaths. This study also highlighted the increase in outbreaks associated with produce. In the 1970s the median number of reported produce-associated outbreaks per year was 2, in the 1980s that number increased to 7, and in the 1990s that number more than doubled to 16. The total percentage of outbreaks associated with produce in the 1970's was 0.7%, while in the 1990's it was 6.0%. In the 190 produce associated outbreaks, there was an etiologic agent identified only 54% (103/190) of the time. Of these, bacteria caused 60%, viruses caused 20%, parasites caused 16%, and chemicals or poisons caused 4%. *Salmonella* was the most common bacterial agent and norovirus was the most common viral agent found on fresh produce.

The increase seen in produce-associated outbreaks may be due to several reasons. Changes in consumer food choices have likely influenced the increase. As more people become conscience of their health, they are changing their diet to include more fruits and vegetables. Sivapalasingham *et al* reported a 24% increase in produce consumption from 1970 to 1997 (64). Also, the globalization of the food supply may have contributed to the increase (52). Fruits and vegetables are now available year round due to imports from other countries.

Produce contamination can occur in a number of ways. Pre harvest sources of contamination may include animal feces in the field. *E. coli* O157:H7 has been shown to be viable in bovine feces for up to 70 days (69). Irrigation and surface run-off waters can also be the cause of contamination in the field (6). Post harvest contamination can occur during processing. For example, if produce is processed in open sheds there is potential for small animals or birds to cause contamination (40). Post harvest contamination can also occur while the produce is being washed. Studies show that if warm produce is soaked in cool water the pressure difference between the core and the surrounding water may allow pathogens in the water to enter the core of the produce, usually through the stem area (4). Other post harvest sources of contamination include contamination by workers and food handlers. Food borne pathogens are easily spread via the fecal-oral route, making transmission from the food handler likely if the food handler does not practice proper hygiene.

Several steps can be taken to help prevent food borne disease caused by contaminated produce. Stricter regulations on foreign farms could decrease outbreaks. In 1996, following an outbreak due to *Cyclosporiasis* in raspberries imported from Guatemala, the FDA banned imports from the country until certain standards were met (34). Similarly, in 2000 and 2002 contaminated cantaloupes were imported from Mexico and caused widespread outbreaks of *Salmonella*. Farms associated in these outbreaks were banned from exporting until further investigation occurred (12). Additionally, to prevent further outbreaks due to contaminated produce, more research needs to be focused on the detection of pathogens on produce. Because there

is an increase in produce related outbreaks, detection methods should be adapted to work efficiently on produce as well as meat and dairy products.

Food borne viruses

More and more reported food borne outbreaks are due to viruses as opposed to bacteria (33). Viruses were probably always a cause of food borne disease; however with recent developments in detection we are now able to confirm the presence of viruses. Previously, those outbreaks may have been recorded as having an unknown causative agent.

Food borne viruses can be divided in to three main categories: 1) viruses that cause gastroenteritis, among these are astrovirus, rotavirus, adenovirus, norovirus (formally Norwalk-like viruses), and SLV (Sapporo-like viruses). 2) viruses that are transmitted through the fecal-oral route, including hepatitis A and hepatitis E. 3) viruses that cause other illnesses, including enteroviruses (46).

There are several differences between food contaminations by a viral pathogen as opposed to a bacterial pathogen. When bacteria are present in food they have the ability to replicate and increase in numbers if the environmental conditions are optimal. Viruses can only replicate in the host and therefore are unable to increase in numbers while in the food. Detection of viruses can be more difficult than the detection of bacteria because many viruses are either difficult to culture or are unable to be cultured in the lab. Also, the number of viral particles present on the food may be very low, hindering detection.

Many foods are at high risk for viral contamination. One group in particular is shellfish. Filter-feeding shellfish have the ability to concentrate viral pathogens in large number (44). Depuration is a process of self-purification used in shellfish production. This process is successful at decreasing the amounts of bacteria present in

shellfish, however the process does not seem to decrease the number of viruses on the shellfish (20, 59, 60). Produce is also often implicated in food borne viral outbreaks. Between 1973 and 1997 there were at least 21 outbreaks of produce-associated food borne viral illness (64). Because a large proportion of produce is eaten raw, there is no cooking step to kill any pathogens that may be present.

Although food borne outbreaks are sometimes seen with rotavirus and astrovirus, these viruses typically affect children as opposed to adults (11). The viruses at the highest risk for food borne transmission are norovirus and hepatitis A virus. The reasons may be due to their extreme stability in the environment and their highly infectious nature (46).

Hepatitis A virus is a small, non-enveloped spherical virus that is about 27-32nm in diameter (57). Initial clinical symptoms of a hepatitis A infection include fever, headache, fatigue, nausea, and abdominal discomfort, followed by symptoms and signs of hepatitis (liver inflammation, jaundice) 1-2 weeks later (7). The virus has an incubation period from 15-50 days, with the average incubation period being around 30 days. This long incubation period can cause many problems with transmission. Hepatitis A is often spread when an infected food handler contaminates the food source (46,70). With such a long incubation period, the food handler may not know he is sick until a month after the infection occurred. In 2001, a food handler at a Massachusetts restaurant likely contaminated the food, which resulted in almost 50 cases of hepatitis A among people who ate at the restaurant (13). The workers' symptoms were not present until after the contamination occurred.

A very large hepatitis A outbreak occurred recently in Pennsylvania (70). Over 500 Pennsylvania residents became ill after eating at a restaurant in Beaver County. Of the 500 sick, at least 124 were hospitalized and 3 died. After an FDA investigation, green onions imported from Mexico were found to be the cause of the outbreak. The vegetables were most likely contaminated either before or during shipping. This is one of the largest reported outbreaks of hepatitis A in the United States. Green onions were a staple ingredient used to make many of the restaurants dishes, this likely contributed to the size of the outbreak. Following the investigation, an import ban was placed on four farms in northern Mexico where the onions likely came from.

The virus most commonly seen in food borne disease is norovirus. It has been estimated, based on surveillance data, that norovirus may account for over 60% of food borne illnesses (52). Norovirus is of fecal origin and many reported outbreaks are due to contaminated water (8, 26, 29) or food (5, 35, 49). One way the food or water source could initially be contaminated is through the hands of an infected person or food handler. Once the initial infection occurs the virus rapidly spreads from person to person via direct contact with contaminated surfaces. The virus can continue to be shed long after physical signs of the disease have disappeared, which further promotes transmission. In some cases, the virus may be shed for up to three weeks, which is long after any physical signs of illness are present in the host (45).

Norovirus was first discovered in 1968 in the small town of Norwalk, Ohio (42). The virus was the cause of a gastroenteritis outbreak in an elementary school. Norovirus is a single stranded RNA virus that can range in size from 27-35 nm. It has

a 1-3 day incubation period with clinical symptoms including a low-grade fever, vomiting, diarrhea, and headache. The infectious dose of norovirus is very low, as low as 10 particles (37). Because the virus is rapidly spread from person-person via contaminated surfaces, outbreaks are usually seen in places with close living conditions for example; nursing homes, hospitals, hotels, and cruise ships (10, 15, 29, 35, 51).

Norovirus belongs to the family *Caliciviridae*. Nomenclature and genetic classification of the virus has posed to be a problem due to the vast genetic diversity of the group. It has been suggested that the genetic diversity may in part be due to point mutations created during the error-prone process of RNA replication (24). To date, four main “genogroups” of norovirus have been described. A genogroup can be defined as “a minimum classification unit consisting of the genetic clusters that reproducibly group together on a distinct branch of a phylogenetic tree and are sufficiently close in both amino acid and nucleotide sequences to be distinguished from genetic clusters falling outside the group” (2). Humans are mainly infected from viruses in either genogroup I or genogroup II. Within genogroup I there are at least seven distinct gene clusters and within genogroup II there are at least eight distinct gene clusters (22).

Due to the great genetic diversity of the virus, detection is difficult. Norovirus was first detected using electron microscopy; however this technology can be quite insensitive (2). In recent years, detection has primarily been done using reverse transcriptase polymerase chain reaction (RT-PCR). The problem with RT-PCR is due to the genetic diverse population of noroviruses there is not one set of primers that

can routinely detect the virus. To further complicate detection, norovirus is unable to be cultured in the lab.

With produce being a food group likely to be contaminated with viral pathogens and with produce associated outbreaks on the rise, this may be an area of food safety to focus on. Better detection methods could help decrease viral contaminants on produce.

Indicator organisms

Indicator organisms are commonly used when the pathogen of interest, for example norovirus, is either unable to be detected or is difficult to detect. *E. coli* and other fecal coliforms are most commonly used when looking for fecal contamination, especially in water sources. The United States Environmental Protection Agency (EPA) has set specific guidelines for coliform testing. There are two types of coliforms tested for, total coliforms and fecal coliforms. Total coliforms are a related group of bacteria that are natural inhabitants of soil, lakes, and rivers. These bacteria are typically not found in ground water. The fecal coliform group is comprised of many different species of bacteria including: *Escherichia*, *Enterobacter*, *Citrobacter*, and *Klebsiella* (47). If total coliforms are found in ground water, there is a potential risk for fecal contamination, including harmful pathogens. If fecal coliforms are detected, that risk is greatly increased (65).

While *E. coli* and coliforms have been the standard for determining fecal contamination in water, they may not be the best choice when it comes to fresh produce. One major reason is that some of the coliform bacteria, for example *Klebsiella*, are commonly found on plants as part of their normal flora and have non-fecal origins (47). Because this organism is part of the plants normal flora, it would not be an ideal indicator for fecal contamination. Also, while *E. coli* does have an exclusive fecal origin, it can be timely and difficult to differentiate *E. coli* from other coliform organisms. Another organism that has been proposed as an indicator is

Enterococcus (39, 43). However, like some other coliforms, not all *Enterococcus* have a fecal origin, making it a poor choice for a fecal indicator organism.

Bacterial indicator organisms are also not an ideal indicator for enteric viruses. Bacteria and viruses behave very differently in the environment and have different survival mechanisms. While most bacteria would be killed by exposure to extreme heat and chlorine, certain viruses would be able to survive these treatments. A study using the MS2 bacteriophage, a type of FRNA bacteriophage, as a surrogate for norovirus found that the bacteriophage was not effectively removed by chlorine washing (17).

FRNA bacteriophages have been proposed as possible fecal indicator organisms. FRNA bacteriophages are viruses that infect and replicate in bacterial hosts by attachment to the F+ or sex pili (53). Their ideal bacterial host is *E. coli*. There are many reasons why we should look at potentially using FRNA bacteriophages as fecal indicators for enteric viruses, including norovirus, instead of the typical fecal coliforms. Mainly, FRNA bacteriophages are similar in size to enteric viruses (27-35nm), they are stable in the environment as are enteric viruses, and they are both resistant to similar treatment processes (1). Also, because the ideal host for the bacteriophage is *E.coli*, which is found in the digestive tract of warm-blooded animals, the bacteriophage itself can usually be found in the digestive tract of warm-blooded animals, making it an ideal candidate as a fecal indicator organism (31).

The correlation between FRNA bacteriophages and enteric viruses has been studied. In 1993, one study looked at the presence of FRNA bacteriophage and

enteric viruses in several different sources of fresh water including: river water, lake water, and recreational water. Each water source was tested for FRNA bacteriophages, thermotolerant coliforms, fecal *Streptococci*, enteroviruses, and enteric viruses. Their overall findings showed there to be a tendency of virus concentrations to be correlated to both the FRNA bacteriophages and the fecal coliforms (31). They suggest that FRNA bacteriophages are a suitable alternative to direct virus detection.

Additional research has been done looking at the use of FRNA bacteriophages as indicator organisms on specific foods including shellfish (19, 56), meat (36, 53), and produce (1, 21). In one study involving shellfish (56), the authors tested oysters for *E. coli*, hepatitis A virus, enterovirus, human adenovirus, and FRNA bacteriophages. Their results show that hepatitis A virus, enterovirus, and human adenovirus were repeatedly detected in oyster samples absent of *E. coli*. They conclude that a viral parameter should be included when looking at indicators for fecal contamination. Another study involving shellfish also concluded that the absence of *E. coli* does not necessarily indicate an absence of potentially pathogenic viruses (19). Similar findings were found in the studies dealing with meat and poultry. In the two studies involving fresh produce, FRNA bacteriophage were detected on produce samples in relatively large number. One study had a 25.3% positive rate (21), while the other reported a 32.5% positive rate (1). Both groups proposed using FRNA bacteriophage in addition to *E. coli* when screening for fecal contamination in fresh produce.

Goals of study

Better detection methods may be one way to help reduce food borne disease. Current methods for determining fecal contamination are not ideal for produce and viral contaminants. These methods need to be adapted or revised to better aid in detection. Our study focused on the detection of norovirus and potential indicator organisms on fresh produce samples. First, we evaluated the effectiveness of five different sets of reverse transcriptase PCR primers from previously published work that were designed for norovirus detection. Second, we screened fresh produce samples for the presence of *E. coli*, *Enterococcus*, and FRNA bacteriophages and looked at their potential for being indicator organisms.

Materials and Methods

Reverse transcriptase PCR evaluation:

Five primer sets were obtained from previously published work: MON primer set (61), SR primer set (2), NVp primer pair (50), NV primer pair (54), and the JV primer pair (67) (table 1). All primers were tested using two positive controls: genogroup I and genogroup II. Positive controls were received in the form of stool samples from the CDC from patients known to be shedding the virus. RNA was extracted from the stool samples for all further analysis. A one-step reverse transcriptase PCR protocol was used for all primers. The reaction mixture contained: 25 ul MasterAmp 2X PCR Premix G (Epicentre Biotechnologies, Madison, WI), 0.5uM of each primer, 0.5ul Rnase Inhibitor (Roche, Indianapolis, IN), 0.09ul AMV-RT (Promega, Madison, WI), and 0.25ul Ampli-Taq (Applied Biosystems, Foster City, CA).

Cycling conditions varied for each primer set depending on their annealing temperatures. For the MON, SR and NVp primer sets the conditions were as follows: 42°C for 60 min, 94°C for 2 min, (94°C 1 min/50°C 1 min 30 sec/60°C 2 min) 40 cycles, 72°C 7 min. For the NV primer set: 42°C for 60 min, 94°C for 2 min, (94°C 30sec/42°C 30 sec/72°C 1 min 30 sec) 40 cycles, 72°C for 7 min. Finally, for the JV primer set: 42°C for 60 min, 94°C for 2 min, (94°C for 1 min/37°C 1 min 30 sec/74°C 1 min), 72°C for 7 min.

All RT-PCR products were examined by electrophoresis on 2% agarose gels. Gels were run at 100 volts for one hour, stained with ethidium bromide, and visualized using ultraviolet light.

Sample collection and processing:

One hundred and eighty fresh produce samples were collected from three local grocery stores. The samples were from 6 groups of produce: celery, carrots, radish, green onions, lettuce, and packaged salad (packaged salad can be defined as any lettuce or vegetables that have been chopped/prepared and sold in plastic bags for immediate use). Thirty samples from each group were analyzed, with 10 samples taken from each store. These groups of produce were picked for several reasons. Mainly, because each of these types of produce are grown either in or closely to the ground increasing their chance of fecal contamination from the soil. Also, several of these varieties of produce have been identified in previous food borne viral outbreaks (46, 64).

A produce wash was prepared for each sample. All produce was purchased the same morning it was to be processed to ensure freshness. Once at the lab, 50g of sample were placed in a Ziploc bag with 100mL of 0.1% peptone water (Bacto Peptone, BD, Franklin Lakes, NJ). The sample was massaged by hand for 5 minutes. All further analysis was done using the produce wash.

FRNA bacteriophage detection:

A spot enrichment assay was used for detection of the bacteriophage. Although the spot enrichment method cannot quantify how many bacteriophage are present on the sample, we chose this method over a quantification method because the levels of bacteriophage expected on the samples are quite low. If a quantification method was used, there is a chance of not detecting the bacteriophage at all. The enrichment step of the spot enrichment method allows for detecting bacteriophages in

small amounts quite easily. The protocol followed was obtained from the United States EPA (66). Briefly, 100ml of produce wash was incubated overnight at 37°C with 5 mL of tryptone enrichment broth (10.0g Bacto peptone, 10.0g yeast extract, 1.0g glucose, 8.0g NaCl₂, and 0.22g CaCl₂, per every 100mL of dH₂O) 1.25mL CaCl₂, and 4mL of a 4 hour culture of the host bacteria (*E. coli* Famp). Ampicillin and streptomycin were added at 1.5 ug/mL to select for only the host bacteria. *E. coli* Famp carries resistant markers for both ampicillin and streptomycin (17). Following incubation, 10ul of the enrichment culture was spotted onto a spot agar dish (1.0g Bacto tryptone, 1.0g yeast extract, 0.1g glucose, 0.8g NaCl₂, 0.022g CaCl₂, and 0.75g Bacto agar per every 100mL of dH₂O. After autoclaving, agar was brought to 45°C in a water bath and 2mL of a four-hour culture of *E. coli* Famp was added for every 100mL of agar. Finally, 1.5 ug/mL of ampicillin and streptomycin were added). The plate was incubated for 24 hours at 37°C (figure 1). After incubation, the plate was examined for lyses zones. MS2 coliphage was used as a positive control.

***E. coli* detection:**

Before finalizing the methods for detecting *E. coli* on the produce samples, we performed a method comparison of two different protocols to determine which protocol would work best. Protocols were modified from previously published work (21, 55). See figure 2 for details of protocols 1 and 2. In order to compare the protocols, we first inoculated two 50g samples of lettuce with two different dilutions (10⁻⁷ and 10⁻⁸) of nalidixic acid resistant *E. coli*. By using nalidixic acid resistant strains of *E. coli* we would be able to determine if the *E. coli* we inoculated the lettuce with was in fact recovered by the methods used for isolation. For both

protocols, the final plates used for isolation were supplemented with 50 ug/mL of nalidixic acid in order to select for the *E. coli* strain used to inoculate the lettuce.

The lettuce samples were washed in a Ziploc bag with 100mL of 0.1% peptone water. This wash was used for further analysis in the method comparison. Protocols were followed as described in figure 2. After examining the final plates used in each protocol, MacKonkey's plates (Difco, Detroit, MI) for protocol 1 and EMB plates (BBL Microbiological Systems, Cockeysville, MD) for protocol 2, we concluded that protocol 2 was more effective at recovering the nalidixic acid resistant *E. coli* from the lettuce samples and would therefore be more beneficial to our study.

Protocol 2 was followed for all *E. coli* detection in our study: 10mL of produce wash was incubated with 10mL double strength Lauryl Sulfate Tryptose (LST) broth (Difco) at 37°C for 48 hours. Broths showing growth after 48 hours were transferred to Brilliant Green Bile (BGB) broth (Difco) and incubated at 37°C for 24 hours. Finally, positive BGB broths were streaked onto EMB (BBL Microbiological Systems) agar for isolation. Up to four presumptive *E. coli* colonies were selected from each plate. Colonies were screened for citrate utilization using Simmons Citrate Agar (BBL Microbiological Systems). All colonies negative for citrate utilization were confirmed to be *E. coli* with Biomeriux API 20 E strips (Biomeriux, France), following manufacturers' instructions.

***Enterococcus* detection:**

In order to detect *Enterococcus*, 10mL of produce wash was incubated in 10mL double-strength Enterococcosel broth (BBL Microbiological Systems) at 37°C for 24 hours. Positive broths were then streaked onto Enterococcosel agar (BBL Microbiological Systems) for isolation (39). Presumptive *Enterococcus* colonies were gram stained.

Results

Reverse transcriptase PCR evaluation

Detection of norovirus through reverse transcriptase PCR was performed using five sets of primers obtained from previously published work. There were varying results among primer sets. Each set was run against two positive controls, RNA from genogroup I and RNA from genogroup II. Of the five sets tested, only the MON primer set and the NV primer set gave a positive result for both control RNA samples (figures 3 and 4). The SR primer set had a positive result with only the RNA from genogroup I (figure 5). Finally, both the NVp110/NVp36 primer set and the JV12/JV13 primer set were negative for both control RNA samples.

FRNA bacteriophage detection:

FRNA bacteriophages were detected on fresh produce samples using the spot enrichment method. After incubation on a spot agar dish, the dish was analyzed for zones of lyses. A zone of lyses can be described as a clearing in the bacterial lawn surrounding the 10ul drop of enrichment culture spotted onto the plate (figure 6). Of the 180 samples of produce tested, 37.2% tested positive for FRNA bacteriophage (67/180). The two groups of produce showing the highest positive rate were radishes and packaged salads; both groups had a 46.6% positive rate (14/30). The group with the next highest positive rate was celery with 43.3% of samples positive (13/30), followed by green onions with a 40% positive rate (12/30). The group with the lowest positive rate was carrots with only 13.3% of samples positive (4/30) (table 2).

There was a difference between FRNA bacteriophage positive rates among the three stores samples were taken from. Store A had the highest rate of detection with

46.6% of samples testing positive for the bacteriophage. Store B was next with a 45% positive rate, followed by store C with only 20% of samples testing positive (figure 7).

***E.coli* detection:**

All produce samples were screened for the presence of *E. coli*. Of the 180 produce samples tested, no samples were positive for *E. coli*.

***Enterococcus* detection:**

All produce samples were screened for *Enterococcus*. Of the 180 samples tested, 17.2% tested positive for *Enterococcus* (31/180). The group of produce with the highest prevalence of *Enterococcus* was radishes with 46.7% positive (14/30). Celery had the next highest rate with 20% testing positive (6/30), followed by green onions with 16.7% testing positive (5/30). Lettuce and packaged salad both had 10% positive (3/30). Finally, no carrots tested positive for *Enterococcus* (table 2).

Discussion

Reverse transcriptase PCR (RT-PCR) has commonly been used for detecting norovirus. However, as shown in our study, it is difficult to find primers that will routinely work. Of the five primer sets tested, only two worked for both of the control RNA samples. However, this is not to say the other three primer sets would not work on RNA taken from different noroviruses. The genetic diversity among the viruses makes it difficult to select primers for RT-PCR. A universal set of primers that has the ability to detect all viruses has yet to be developed.

The two primer sets that did work, MON and NV, could potentially be used for screening for norovirus. However, more samples would need to be tested to better determine their sensitivity.

Due to the inconsistencies in detection, we did not pursue using RT-PCR detection any further in this study. Similar studies have also found that detection by RT-PCR is difficult and can be inconclusive (54). The number of viral particles expected on produce is very low, which would make RT-PCR detection difficult even if a universal set of primers did exist. An indicator organism, possibly FRNA bacteriophage, would be more efficient than RT-PCR in assessing fecal contamination on produce.

A relatively high number of samples were positive for FRNA bacteriophage, 37.2% total. These findings are similar to what other studies have found. Allwood *et al* found 32.5% of produce tested positive for the bacteriophage and Endley *et al* found 25.3% of produce tested positive (1, 21).

It is not surprising that packaged salad was among the group with the highest detection rate. One possible reason for this is packaged salad is handled more than other groups of produce. The chopping/slicing/preparing of the product allows for more opportunities for handling and possibly more opportunities for cross-contamination. This has also been seen in similar studies (1). In another study (21), carrot samples were tested along various steps in harvesting for the presence of FRNA bacteriophage. The field, the truck on the way to processing, and the processing shed were all tested. The processing shed, where workers repeatedly handled the carrots, had the highest number of positive samples compared to the other two locations.

The results obtained here also demonstrate the ease at which FRNA bacteriophage can be detected from produce samples. Even if the bacteriophage is present in low levels on the produce, the enrichment step of the detection protocol allows for replication. The methods used are quick, require minimum lab equipment, and are easy to perform. In contrast, RT-PCR requires expensive thermal cyclers and gel electrophoresis equipment. RT-PCR also requires more advanced laboratory skills than the FRNA bacteriophage detection methods.

E. coli was not detected with the same ease as FRNA bacteriophages. After selecting a protocol we thought would be best for detection, *E. coli* was not found on any of the produce samples. Other bacteria were shown to be present, but no *E. coli* was found. As confirmed by Biomerieux API 20 E strips, bacteria such as *Klebsiella*, *Citrobacter*, and *Enterobacter* were found on the samples.

Although we found no *E. coli*, similar studies have found very low levels (1,21). There may be several reasons why *E. coli* was not detected. First, the levels of *E. coli* present on the produce may have been extremely low, making detection difficult. Second, it is possible that the other bacteria present, *Klebsiella*, *Citrobacter*, and *Enterobacter*, may have been causing some competition and inhibited the growth of *E. coli*. Finally, some produce washes were frozen at -80°C for several weeks before being analyzed for *E. coli*. While this step was supposed to help preserve the bacteria, it may have actually had the opposite effect.

Enterococcus was found at a rate of 17.2%, with radishes having the most number of positive samples. In a study that looked at the antimicrobial resistance patterns of *Enterococcus* isolated from produce, *Enterococcus* was isolated from 47% of the samples (39). Although the goals of this study were not to look at *Enterococcus* as a fecal indicator, it demonstrates how often this bacterium is isolated from produce. *Enterococcus* does not always have a fecal origin; it is also found as normal flora in soil and plants (32). Therefore, detection of *Enterococcus* on produce does not necessarily mean there has been fecal contamination.

There was a difference between FRNA bacteriophage positive rates from store to store. Stores A and B had relatively the same positive rates with 46.6% and 45%, respectively. Store C had only 20% positive. The reason for this may be due to the individual stores handling and processing guidelines. It may come down to contamination by food handlers. For FRNA bacteriophage detection within each produce group, there did not appear to be a large difference between the numbers of positive samples obtained from each store, only that store C generally had the least

amount of positives. However, for *Enterococcus* detection, all 6 positive celery samples and all 3 positive lettuce samples were from store B. Because little is known about what happens to the produce before it reaches the displays, it is hard to say why we see this trend. The most likely cause is contamination from workers or cross-contamination with other foods.

Conclusions

We conclude that FRNA bacteriophage has the potential at being an ideal indicator organism for detecting norovirus and possibly other viruses on fresh produce. Traditional indicators such as *E.coli* and *Enterococcus* may not be the best choice when it comes to produce or viral pathogens. Further work would need to be done to better determine the correlation between FRNA bacteriophage and norovirus. However, this preliminary data demonstrates the possibility of at least including FRNA bacteriophage along with traditional indicator organisms in detecting fecal contamination on fresh produce.

Table 1: Primer sequences used in RT-PCR evaluation

Primer name	Sequence	Expected product Length
Mon 431	5' TGG ACI AGR GGI CCY AAY CA 3'	231bp
Mon 432	5' TGG ACI CGY GGI CCY AAY CA 3'	
Mon 433	5' GAA YCT CAT CCA YCT GAA CAT 3'	
Mon 434	5' GAA SCG CAT CCA RCG GAA CAT 3'	
SR 33	5' TGT CAC GAT CTC ATC ATC ACC 3'	123 bp
SR 46	5' TGT AAT TCC ATC GCC CAC TGG 3'	
SR 48	5' GTG AAC AGC ATA AAT CAC TGG 3'	
SR 50	5' GTG AAC AGT ATA AAC CAC TGG 3'	
SR 52	5' GTG AAC AGT ATA AAC CAT TGG 3'	
NVp110	5' ACD ATY TCA TCA CCA TA 3'	398 bp
NVp36	5' ATA AAA GTT GGC ATH AAC A 3'	
NV51	5' GTT GAC ACA ATC TCA TCA TC 3'	206 bp
NV3	5' GCA CCA TCT GAG ATG GAT GT 3'	
JV 12	5' ATA CCA CTA TGA TGC AGA TTA 3'	327 bp
JV 13	5' TCA TCA TCA CCA TAG AAA GAG 3'	

Table 2: Prevalence of FRNA bacteriophage, *E. coli*, and *Enterococcus* in fresh produce samples.

Prevalence of FRNA Bacteriophage, <i>E. coli</i> , and <i>Enterococcus</i> no. pos samples (%)						
Produce Variety	Store	n	FRNA Bacteriophage	<i>E.coli</i>	<i>Enterococcus</i>	
Celery	A	10	5 (50)	0 (0)	0 (0)	
	B	10	5 (50)	0 (0)	6 (60)	
	C	10	3 (30)	0 (0)	0 (0)	
	total	30	13 (43.3)	0 (0)	6 (20)	
Carrot	A	10	3 (30)	0 (0)	0 (0)	
	B	10	1 (10)	0 (0)	0 (0)	
	C	10	0 (0)	0 (0)	0 (0)	
	total	30	4 (13.3)	0 (0)	0 (0)	
Radish	A	10	5 (50)	0 (0)	6 (60)	
	B	10	6 (60)	0 (0)	4 (40)	
	C	10	3 (30)	0 (0)	4 (40)	
	total	30	14 (46.6)	0 (0)	14 (46.7)	
Green Onion	A	10	5 (50)	0 (0)	1 (10)	
	B	10	2 (20)	0 (0)	2 (20)	
	C	10	5 (50)	0 (0)	2 (20)	
	total	30	12 (40)	0 (0)	5 (16.7)	
Lettuce	A	10	4 (40)	0 (0)	0 (0)	
	B	10	4 (40)	0 (0)	3 (30)	
	C	10	2 (20)	0 (0)	0 (0)	
	total	30	10 (33.3)	0 (0)	3 (10)	
Packaged Salad	A	10	6 (60)	0 (0)	1 (10)	
	B	10	6 (60)	0 (0)	0 (0)	
	C	10	2 (20)	0 (0)	2 (20)	
	total	30	14 (46.6)	0 (0)	3 (10)	
Grand Total		180	67 (37.2)	0 (0)	31 (17.2)	

FIGURE 1: Spot enrichment assay for detecting FRNA bacteriophage (66).

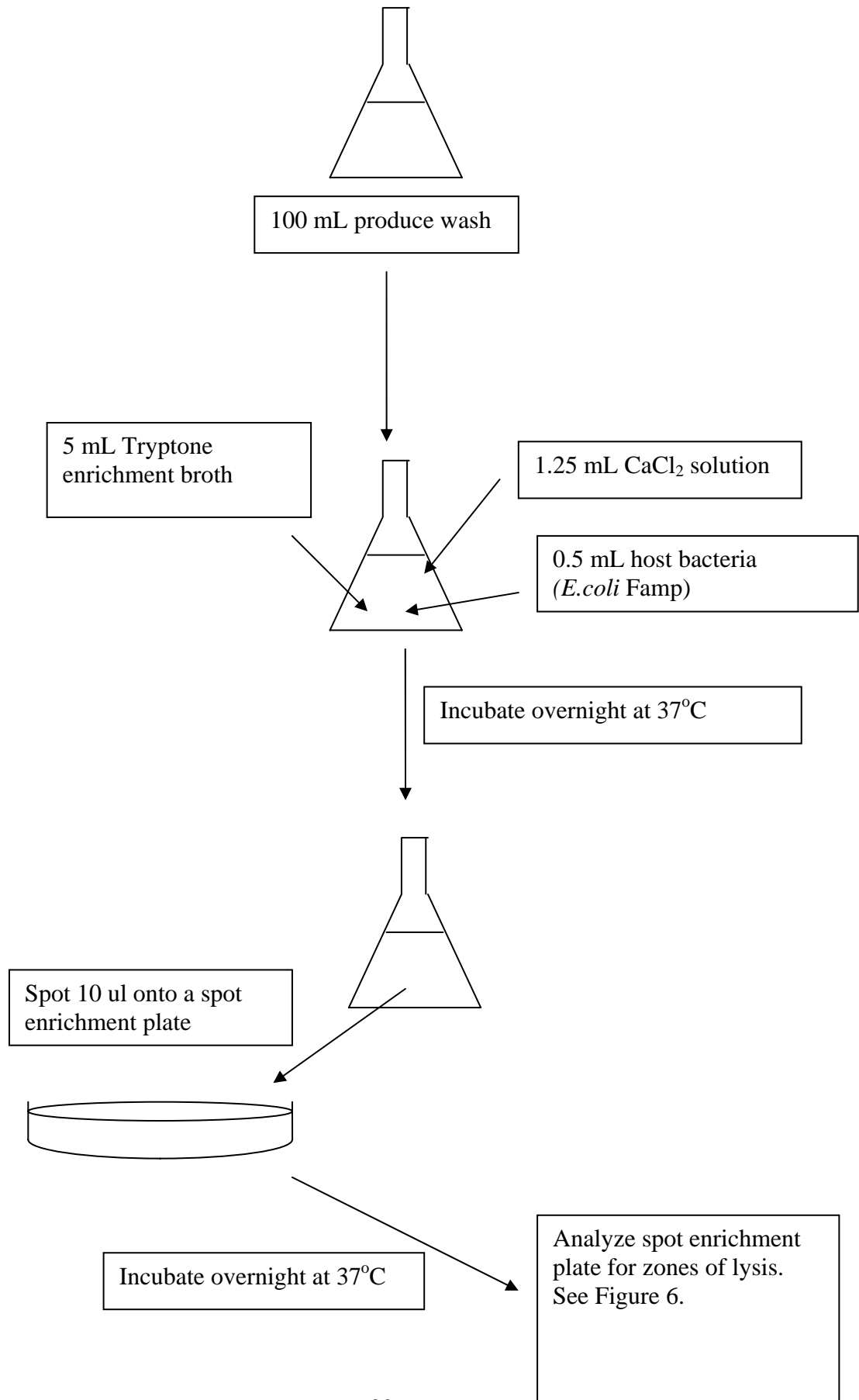
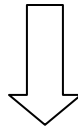


FIGURE 2: Protocols 1 and 2 for *E. coli* detection (21,55).

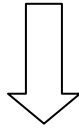
Protocol 1:

10 mL produce wash + 10 mL double strength Lauryl Sulfate Tryptone (LST) broth



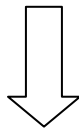
Incubate for 24 hours at 37°C

If LST broth is positive, incubate 1 mL positive LST broth in 5mL EC broth



Incubate for 24 hours at 37°C

Spread 100ul of EC broth onto MacKonkey plate

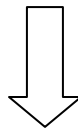


Incubate for 24 hours at 37°C

Look for presumptive *E.coli* colonies.

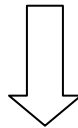
Protocol 2:

10 mL produce wash + 10 mL double strength Lauryl Sulfate Tryptone (LST) broth



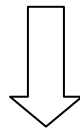
Incubate for 24 hours at 37°C

If LST broth is positive, incubate 1 mL LST broth in 5mL Brilliant Green Broth



Incubate for 24 hours at 37°C

Spread 100ul of Brilliant Green Broth into EMB plates



Incubate for 24 hours at 37°C

Look for presumptive *E. coli* colonies

Figure 3: Amplification of the genogroup I and genogroup II control RNA using the MON primer set. Lane 1: 1Kb ladder, Lane 2: genogroup I RNA, Lane 3: genogroup II RNA, Lane 4: negative control.

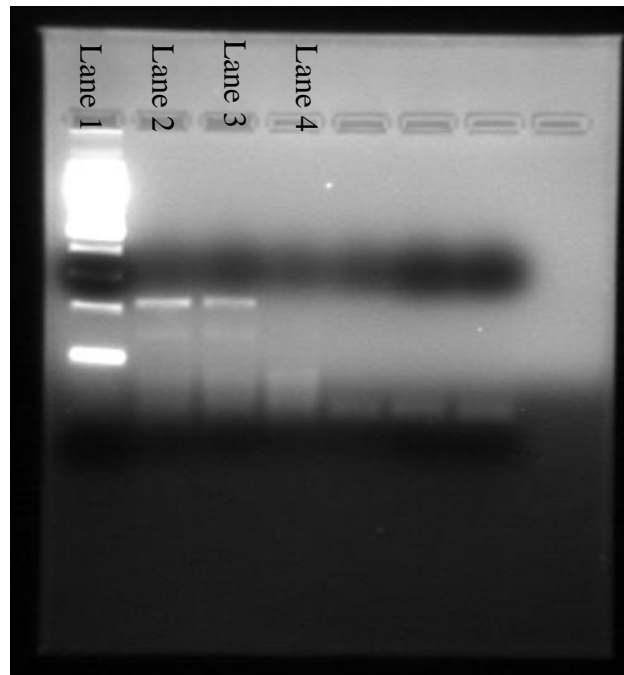


Figure 4: Amplification of the genogroup I and genogroup II control RNA using the NV51/NV3 primer pair. Lane 1: 1Kb ladder, Lane 2: genogroup I RNA, Lane 3: genogroup II RNA, Lane 4: Negative control.

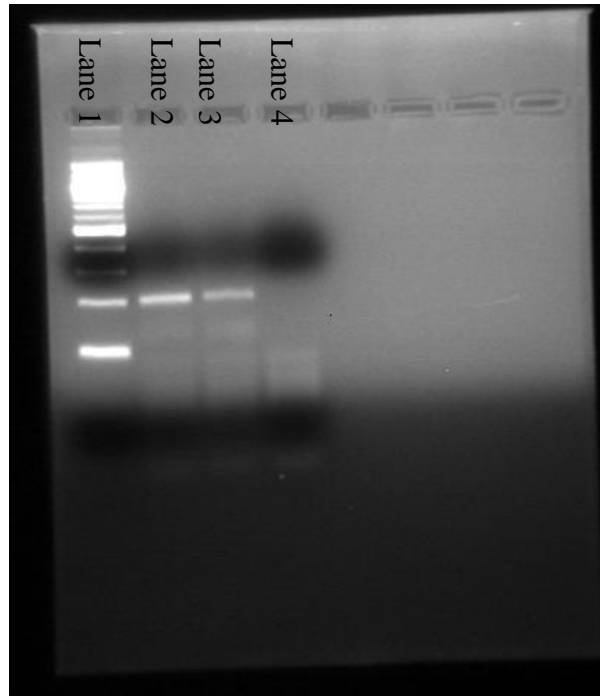


Figure 5: Amplification of the genogroup I control RNA using SR primer set. Lane 1: 1Kb ladder, Lane 2: genogroup I control RNA, Lane 3: genogroup II RNA, Lane 4: negative control.

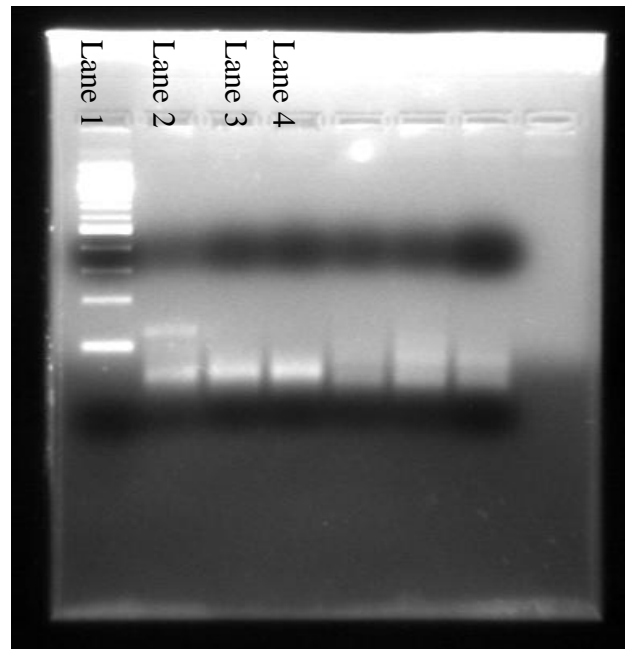


Figure 6: Spot enrichment assay for detecting FRNA bacteriophage. Spot agar dish showing lyses zones for a celery and a lettuce sample. MS2 used as positive control.

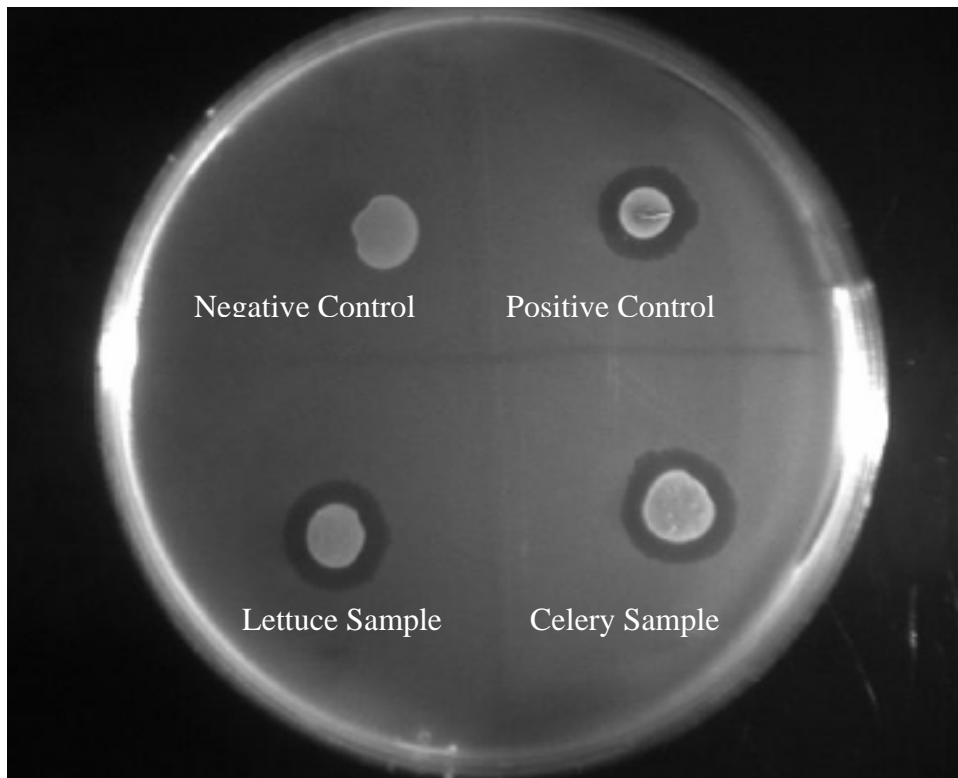
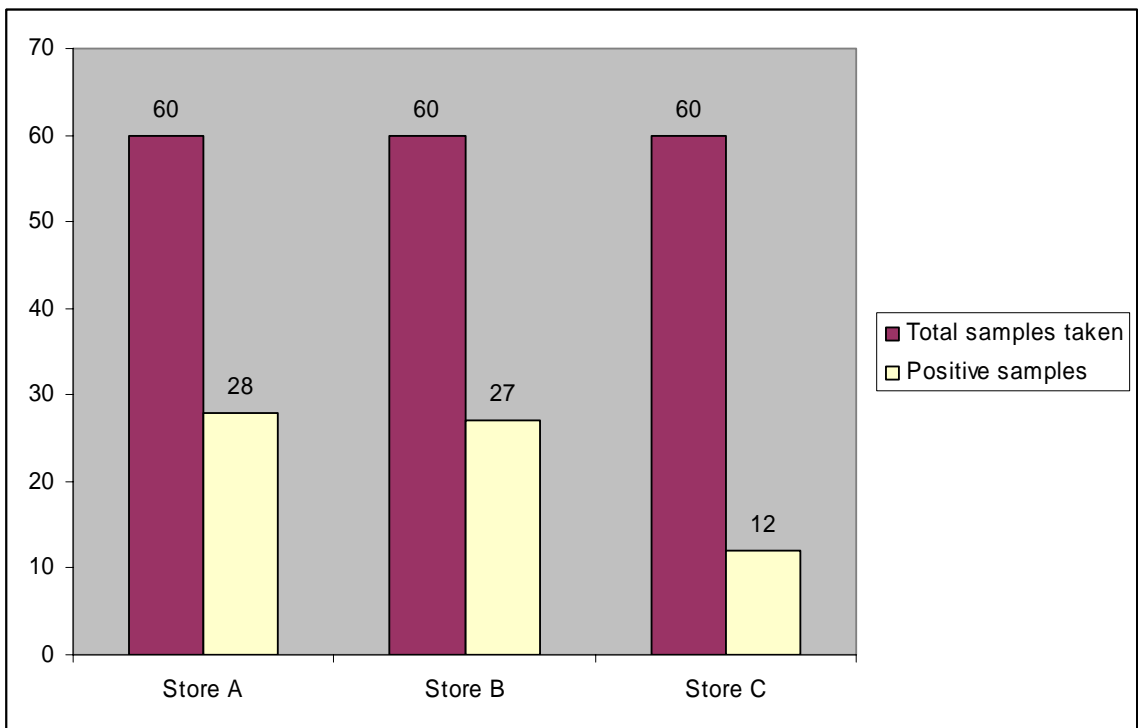


Figure 7: Number of produce samples positive for FRNA bacteriophages, separated by store.



References

1. Allwood, P.B., Y.S. Malik, S. Maherchandani, K. Vought, L.A. Johnson, C. Braymen, C. Hedberg, and S.M. Goyal. 2004. Occurrence of *Escherichia coli*, norovirus, and F-specific coliphages in fresh market-ready produce. *J. Food Prot.* 67:2387-2390.
2. Ando, T., S.S. Monroe, J.R. Gentsch, Q. Jin, D.C. Lewis, R.I. Glass. 1995. Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-Like Viruses) by reverse- transcription PCR and southern hybridization. *J. Clin. Microbiol.* 33:64-71.
3. Ando, T., J.S. Noel, and R.L. Fankhauser. 2000. Genetic classification of “Norwalk-Like Viruses”. *J. Infect. Dis.* 181:S336-48.
4. Bartz, J., R.K. Showalter. 1981. Infiltration of tomatoes by aqueous bacterial suspensions. *Phytopathology* 71:515-518.
5. Bearet, C., A. Baumgartner, J. Schluep. 2003. Virus-contaminated oysters, a three-month monitoring of oysters imported to Switzerland. *Appli. Environ. Microbiol.* 59:2292-2297.
6. Beuchat, L.R., J.H. Ryu. 1997. Produce handling and processing practices. *Emerg. Infect. Dis.* 3:459-465.
7. Boughton, C.R., R.A. Hawkes, D.R. Schroeter, V. Ferguson, N.I. Lehmann, J. Harlor, V. Watson, M. Cumpston. 1982. Viral hepatitis: a four year hospital and general-practice study in Sydney. Epidemiological features, natural history, and laboratory findings. *Med. J. Aust.* 1:113-119.

8. Brugha, R.I., B. Vipond, M.R. Evans, Q.D. Sandifer, R.J. Roberts, R.L. Salmon, E.O. Caul, A.K. Mukerjee. 1991. A Community outbreak of food-borne small round-structured virus gastroenteritis caused by a contaminated water supply. *Epidemiol. Infect.* 122:145-154.
9. Bryan, F.L. Diseases transmitted by foods. Atlanta: Centers for Disease Control: 1982.
10. Calderon-Margalit, R., R. Sheffer, T. Halperin, N. Orr, D. Cohen, T. Shohat. 2005. A large-scale gastroenteritis outbreak associated with norovirus in nursing homes. *Epidemiol. Infect.* 133:35-40.
11. Centers for Disease Control. 1990. Viral agents of gastroenteritis: public health importance and outbreak management. *Morbid. Mortal. Wkly. Rep.* 39(RR-5):1-24.
12. Centers for Disease Control. 2002. Multistate outbreaks of *Salmonella* serotype Poona infections associated with eating cantaloupe from Mexico-United States and Canada, 2000-2002. *Morbid. Mortal. Wkly. Rep.* 51:1044-7.
13. Centers for Disease Control. 2003. Food borne transmission of Hepatitis A – Massachusetts, 2001. *Morbid. Mortal. Wkly. Rep.* 52(24): 565-567.
14. Centers for Disease Control. 2005. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food – 10 sites, United States, 2004. *Morbid. Mortal. Wkly. Rep.* 54:352-356.
15. Chessbrough, J.S., J. Green, C. Gallimore, P. Wright, D. Brown. 2000. Widespread environmental contamination with NLV detected in a prolonged hotel outbreak of gastroenteritis. *Epidemiol. Infect.* 125:92-98.

16. Cole, D., S.C. Long, M.D. Sobsey. 2003. Evaluation of F+ RNA and DNA coliphages as source-specific indicators of fecal contamination in surface waters. *Appl. Environ. Microbiol.* 69:6507-6514.
17. Dawson, D.J., A. Paish, L.M. Staffell, I. J. Seymour, and H. Appleton. 2005. Survival of viruses on fresh produce, using MS2 as a surrogate for norovirus. *J. Appl. Microbiol.* 98:203-209.
18. Debartolomeis, J., V.J. Cabelli. 1991. Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific bacteriophages. *Appl. Environ. Microbiol.* 57:1301-1305.
19. Dore, W.J., K. Henshilwood, and D.N. Lees. 2000. Evaluation of F-specific bacteriophage as a candidate human enteric virus indicator for bivalve molluscan shellfish. *Appl. Environ. Microbiol.* 66:1280-1285.
20. Dore, W.J., D.N. Lees. 1995. Behavior of *Escherichia coli* and male-specific bacteriophages in environmentally contaminated bivalve mollusks before and after depuration. *Appl. Environ. Microbiol.* 61:2830-2834.
21. Endley, S., L. Lu, E. Vega, M.E. Hume, S.D. Pillai. 2003. Male-specific coliphages as an additional fecal contamination indicator for screening fresh carrots. *J. Food Prot.* 66:88-93.
22. Fankhauser, R.L., S.S. Monroe, J.S. Noel, C.D. Humphrey, J.S. Bresee, U.D. Parashar, T. Ando, R.I. Glass. 2002. Epidemiologic and molecular trends of "Norwalk-Like Viruses" associated with outbreaks of gastroenteritis in the United States. *J. Infect. Dis.* 186:1-7.

23. Foodborne illnesses: ten least wanted foodborne pathogens. April 2003.
retrieved on October 18, 2005 from the world wide web:
<http://www.fightbac.org/10least/cfm>.
24. Gallimore, C.I., J. Green, D. Lewis, A.F. Richards, B.A. Lopman, A.D. Hale, R. Eglin, J.J. Gray, and D.W.G. Brown. 2004. Diversity of noroviruses cocirculating in the north of England from 1998 to 2001. *J. Clin. Microbiol.* 42:1396-1401.
25. Gaze, J. 2005. Microbiological aspects of thermally processed foods. *J. Appl. Microbiol.* 98:1381-1386.
26. Gray, J.J., J. Green, C. Cunliffe, C. Gallimore, J.V. Lee, K. Neal, D.W. Brown. 1997. Mixed genogroup SRSV infections among a party of canoeists exposed to contaminated recreational water. *J. Med. Virol.* 52:425-429.
27. Green, J., C.I. Gallimore, J.P. Norcott, D.W.G. Brown. 1995. Broadly reactive reverse transcriptase polymerase chain reaction for the diagnosis of SRSV-associated gastroenteritis. *J. Med. Virol.* 47:392-398.
28. Greene, S.R., C.L. Moe, L.A. Jaykus, M. Cronin, L. Grosso, P. van Aarle. 2003. Evaluation of the Nuclisens Basic Kit assay for detection of Norwalk virus RNA in stool specimens. *J. Virol. Meth.* 180:123-131.
29. Hafliger, D., P. Hubner, J. Luthy. 2000. Outbreak of viral gastroenteritis due to sewage contaminated drinking water. *Int. J. Food Microbiol.* 97:179-186.
30. Hardnett, F.P., R.M. Hoekstra, M. Kennedy, L. Charles, F.J. Angulo. 2004. Emerging infections program FoodNet working group. Epidemiologic issues

in study design and data analysis related to FoodNet activities. *Clin. Infect. Dis.* 38(Suppl. 3):S121-S126.

31. Havelaar, A.H., M. van Olphen, Y.C. Drost. 1993. F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. *Appl. Environ. Microbiol.* 59:2956-2962.
32. Hayes, J.R., L.L. English, P.J. Carter, T. Proescholdt, K.Y. Lee, D.D. Wagner, D.G. White. 2003. Prevalence of antimicrobial resistance of *Enterococcus* species isolated from retail meats. *Appl. Environ. Microbiol.* 69:7153-7160.
33. Hedberg, C., K. MacDonald, M. Osterholm. 1994. Changing epidemiology of food-borne disease: a Minnesota perspective. *Clin. Infect. Dis.* 18:671-682.
34. Herwaldt, B.L., M.L. Ackers. 1997. An outbreak in 1996 of cyclosporiasis associated with imported raspberries. The Cyclospora Working Group. *N. Engl. J. Med.* 336:1548-1556.
35. Herwaldt, B.L., J.F. Lew, C.L. Moe, D.C. Lewis, C.D. Humphrey, S.S. Monroe, E.W. Pon, and R.I. Glass. 1994. Characterization of a variant strain of Norwalk virus from a food-borne outbreak of gastroenteritis on a cruise ship in Hawaii. *J. Clin. Microbiol.* 32:961-866.
36. Hsu, F.C., Y.S.C. Shieh, M.D. Sobsey. 2002. Enteric bacteriophages as potential fecal indicators in ground beef and poultry meat. *J. Food Prot.* 65:93-99.
37. Jay, J.M. 1991. *Modern Food Microbiology*, 4th edition. Chapman and Hall, New York.

38. Jaykus, L.A. 1997. Epidemiology and detection as options for control for viral and parasitic foodborne disease. *Emerg. Infect. Dis.* 3:529-538.
39. Johnston, L.M., L.A Jaykus. 2004. Antimicrobial resistance of *Enterococcus* species isolated from produce. *Appl. Environ. Microbiol.* 70:3133-3137.
40. Jones, F., P. Smith, D.C. Watson. 1978. Pollution of a water supply catchments by breeding gulls and the potential environmental health implications. *J. Inst. Water Environ. Manage.* 32:469-82.
41. Kapikian, A.Z. 2000. The discovery of the 27nm Norwalk Virus: an historic perspective. *J. Infect. Dis.* 181(suppl 2): S295-S302.
42. Kapikian, A.Z., R. G. Wyatt, R. Dolin, T.S. Thornhill, A.R. Kalica, R.M. Chanock. 1972. Visualization by immune electron microscopy of a 27 nm particle associated with acute infectious nonbacterial gastroenteritis. *J. Virol.* 10:1075-1081.
43. Kinzelman, J., C. Ng, E. Jackson, S. Gradus, R. Bagley. 2003. *Enterococci* as indicators of Lake Michigan recreational water quality: Comparison of two methodologies and their impacts on public health regulatory events. *Appl. Environ. Microbiol.* 69:92-96.
44. Kohn, M., T. Farley, T. Ando, M. Curtis, S. Wilson, Q. Sin, S. Monroe, R. Baron, L. McFarland, R. Glass. 1995. An outbreak of Norwalk virus gastroenteritis associated with eating raw oysters. *J. Am. Med. Assoc.* 273:466-471.
45. Koopmans, M., E. Duizer. 2004. Foodborne viruses: and emerging problem. *Int. J. Food Microbiol.* 90:23-41.

46. Koopmans, M., C.H. von Bonsdorff, J. Vinje, D. de Medici, S. Monroe. 2002. Foodborne viruses. *FEMS Microbiol. Rev.* 26:187-205.
47. Lang, M.M., S.C. Ingham, B.H. Ingham. 1999. Verifying apple cider plant sanitation and hazard analysis critical control point programs: choice of indicator bacteria and testing methods. *J. Food Prot.* 62:887-893.
48. Leggitt, P.R., L.A. Jaykus. 2000. Detection methods for human enteric viruses in representative foods. *J. Food Prot.* 63:1738-1744.
49. LeGuyader F.S., C. Mittelhozer, L. Haugarreau, K.O. Hedlund, R. Alsterlund, M. Pommepuy, L. Svensson. 2004. Detection of norovirus in raspberries associated with a gastroenteritis outbreak. *Int. J. Food Microbiol.* 97:179-186.
50. LeGuyader, F., F.H. Neill, M.K. Estes, S.S. Monroe, T. Ando, R.L. Atmar. 1996. Detection and analysis of a small round-structured virus strain in oysters implicated in an outbreak of acute gastroenteritis. *Appl. Environ. Microbiol.* 62:4268-4272.
51. Lopman, B.A., M.H. Reacher, I.B. Vipond, J. Sarang, D.W. Brown. 2004. Clinical manifestation of norovirus gastroenteritis in health care settings. *Clin. Infect. Dis.* 39:318-324.
52. Mead, P.S., L. Stutsker, V. Dietz, L.F. McCaig, J. S. Bresee, C. Shapiro, P.M. Griffin, R.V. Tauxe. 1999. Food-related illness and death in the US. *Emerg. Infect. Dis.* 5:607-625.
53. Miller, A.J., B.S. Eblen, A. Oser, W. Burkhardt III. 1998. Application and evaluation of male-specific bacteriophage as a process integrity or faecal

- contamination indicator in a pork slaughterhouse environment. *J. Appl. Microbiol.* 85:898-904.
54. Moe, C.L., J. Gentsch, T. Ando, G. Grohmann, S.S. Monroe, X. Jiang, J. Wang, M.K. Estes, Y. Seto, C. Humphrey, S. Stine, R.I. Glass. 1994. Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis. *J. Clin. Microbiol.* 32:642-648.
55. Mukherjee, A., D. Speh, E. Dyck, F. Diez-Gonzalez. 2004. Preharvest evaluation of coliforms, *Escherichia coli*, *Salmonella*, and *Escherichia coli* O157:H7 in organic and conventional produce grown by Minnesota farmers. *J. Food Prot.* 67:894-900.
56. Muniain-Mujika, I., M. Calvo, F. Lucena, R. Girones. 2003. Comparative analysis of viral pathogens and potential indicators in shellfish. *Int. J. Food Microbiol.* 83:75-85.
57. Najarian, R., D. Caput, W. Gee, S.J. Potter, A. Renard, J. Merryweather, G. VanNest, D. Dina. 1985. Primary structure and gene organization of human hepatitis A virus. *Proc. Natl. Acad. Sci. USA.* 82:2627-2631.
58. "Pathogen Reduction: Hazard Analysis and Critical Control Point (HACCP) Systems; Final Rule." Federal Register. 61(July 25, 1996).
59. Power, U.F., J.K. Collins. 1990. Tissue distribution of a coliphage and *Escherichia coli* in mussels after contamination and depuration. *Appl. Environ. Microbiol.* 55:1386-1390.
60. Richards, G.P. 1988. Microbial purification of shellfish: a review of depuration and relaying. *J. Food. Prot.* 51:218-251.

61. Richards, G.P., M.A. Watson, R.L. Fankhauser, S.S. Monroe. 2004. Genogroup I and II noroviruses detected in stool samples by real-time reverse transcriptase PCR using highly degenerate universal primers. *Appl. Environ. Microbiol.* 70:7179-7184.
62. Rose, B.E., W.E. Hill, R. Umholtz, G.M. Ransom, W.O. James. 2002. Testing for *Salmonella* in raw meat and poultry products collected at federally inspected establishments in the United States, 1998 through 2000. *J. Food Prot.* 65:937-947.
63. Sair, A.I., D.H. D'Souza, C.L. Moe, L.A. Jaykus. 2002. Improved detection of human enteric viruses in foods by RT-PCR. *J. Virol. Meth.* 100:57-69.
64. Sivapalasingam, S., C.R. Friedman, L. Cohen, R.V. Tauxe. 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *J. Food Prot.* 67:2342-2353.
65. USEPA. 2001. A small systems guide to the total coliform rule. Office of Water. EPA. 816-R-01-017A. Environmental Protections Agency, Washington D.C.
66. USEPA. 2001. Manual of Virology Methods. Office of Research and Development. EPA 600/4-84/013. Environmental Protections Agency, Washington D.C.
67. Vinje, J., M.P.G. Koopmans. 1996. Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in the Netherlands. *J. Infect. Dis.* 174:610-615.

68. Vinje, J., H. Vennema, L. Maunula, C.H von Bonsdorff, M. Hoehne, E. Schreier, A. Richards, J. Green, D. Brown, S.S. Beard, S.S. Monroe, E. de Bruin, L. Svensson, M.P.G. Koopmans. 2003. International collaborative study to compare reverse-transcriptase PCR assays for detection and genotyping of noroviruses. *J. Clin. Microbiol.* 41:1423-1433.
69. Wang, G., T. Zhao, M.P. Doyle. 1996. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. *Appl. Environ. Microbiol.* 62:2567-2570.
70. Wheeler C., T.M. Vogt, G.L. Armstrong, G. Vaughan, A. Weltman, O.V. Nainan, V. Dato, G. Xia, K. Waller, J. Arnon, T.M. Lee, A. Highbaugh-Battle, C. Hembree, S. Evenson, M.A. Ruta, I.T. Williams, A.E. Fiore, B.P. Bell. 2005. An outbreak of hepatitis A associated with green onions. *N. Engl. J. Med.* 353:890-897.