

**EFFECTS OF BROAD-SPECTRUM FERTILIZERS ON HUMAN
PICORNAVIRUSES**

by

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ABSTRACT

The presence of disease-causing microorganisms (viruses, bacteria, fungi, and protozoa) on farmlands poses a serious threat to public health. Pathogens can cause surface water contamination by many processes, such as runoff, groundwater contamination through leaching, and food contamination through direct contact or irrigation with contaminated water. This is a critical national issue because of the widespread agricultural use of manures/biosolids, the growing problem of emerging viral diseases, and the limited research base on interactions between viruses in water with soils and food crops. It has been estimated that nearly 80 million dry tons of solid manure are generated annually by the beef, dairy, swine, and poultry industries in the U.S. Land application remains by far the most common and economic method to beneficially re-use these by-products of animal production. Properly treated manure is an effective and safe fertilizer, but untreated or improperly treated manure may contain pathogens that can reach fresh produce in the field, nearby surface waters and leach to ground waters; it is these affected waters that will be used for irrigation and for pesticide applications.

Although viruses are not the only pathogens of concern, they are much smaller in size than other enteric pathogens, and can move better through the subsurface. Viruses also tend to survive longer than bacteria and protozoa in the environment and therefore pose greater threats for groundwater and produce contamination. Furthermore, viruses have been shown to be responsible for approximately 80% of waterborne disease outbreaks for which infectious agents were identifiable and 75% of the estimated cases of foodborne illness.

Due to the recent attention given to foodborne disease outbreaks, especially with lettuce, there is a renewed desire to understand the origin of plant contamination by viruses. Transmission of human pathogens to plants through contaminated irrigation water and contaminated hands has been documented under both laboratory and field conditions. Viruses have previously been shown to survive on the surface of vegetables for more than 2 months under suitable conditions with the potential for survival for over 2 years based on laboratory calculations of the field environment, outlasting the normal shelf-life of some products.

Considerable research has been devoted to the fate and transport of viruses in soils; there is evidence that viruses can move considerable distances in specific soil layers. Such findings have raised concerns about surface and ground water contamination. Generally, viruses have been found to survive longer under moist as compared to dry conditions. In addition, when viruses are associated to a solid surface, they are generally protected from being inactivated; however, association with certain metal and metal oxides has been found to reduce virus survival.

The route of viral contamination often goes unidentified for a majority of foodborne outbreaks involving fresh produce. Nearly 75% of these outbreaks are related to domestically grown produce with less than 10% from imported produce and the rest unknown. As produce consumption has increased with increasing focus on a healthy diet, foodborne illness associated with these foods has as well. Viral contamination of produce has been associated with various types of fruits, leafy vegetables, and herbs. Viruses affect large numbers of people and different food products; the CDC estimates the incidence of foodborne illness attributed to fruit and vegetable consumption at 3 million cases in the U.S. annually.

Chapter 1

REVIEW OF LITERATURE

1.1 Foodborne Outbreaks in the United States

It is estimated that over 200 diseases are transmitted through food, which includes agents that are viral, bacterial, parasitic, toxic, metals, and prions (Bryan 1982, Mead et al. 1999). These diseases range in severity from mild to life-threatening. In the United States, foodborne diseases have been implicated in 6 – 81 million illnesses per year with roughly 9,000 deaths (Todd 1989). With the improvement of technology have come more accurate estimates.

Unfortunately, due to underreporting, it is believed that these estimates are low. Mild cases of foodborne illness that result in gastroenteritis do not generally require hospitalization, and are therefore not detected during routine surveillance. In addition, many foodborne pathogens can also be transmitted through water and from person-to-person contact, which may complicate pinpointing the source of illness. The cause of illness may also have not yet been identified; many pathogens, such as *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Cyclospora cayetanensis* were not recognized as causes of foodborne illness 20 years ago (Mead et al. 1999).

In a study conducted by Mead et al. (1999), the number of food-related illnesses and deaths from known pathogens in the United States were estimated and adjusted for underreporting. Estimates reported by Chalker and Blaser (1988) suggest that the degree of underestimation may be as great as 38-fold for cases of *Salmonella*.

Another study conducted by Hedberg et al. (1997) suggested that reported cases of *E. coli* O157:H7 may be almost 20-fold higher than what is actually reported. Based on these studies, underestimations were accounted for by a factor of 38 for pathogens that cause nonbloody diarrhea, a factor of 20 for pathogens that cause bloody diarrhea, and a factor of 2 for pathogens that cause severe illness (Mead et al. 1999). The reported cases were documented, taken from passive surveillance systems (National Notifiable Disease Surveillance System), or taken from active surveillance systems (FoodNet). Only cases involved in outbreaks were reported. Sporadic cases were not included in this study; it is believed that if sporadic cases were included, the total number of cases would be ten times the current estimates.

After estimating the number of cases caused by each pathogen, Mead et al. (1999) calculated the percentage of illness attributed to foodborne transmission. The total number of cases was then multiplied by this percentage to derive the total number of illnesses. Of the 38.6 million cases estimated in this study, 5.2 million were attributed to bacteria, 2.5 million to parasites, and 30.9 million to viruses. Thirty-six percent of the 38.6 million cases are caused by foodborne transmission; 30% are caused by bacteria, 3% by parasites, and 67% by viruses.

In addition to estimating the total number of foodborne illnesses, the number of hospitalizations due to foodborne transmission was also estimated by multiplying the number of reported cases by present hospitalization rates in previous studies (Mead et al. 1999). Assuming that not all hospitalized illnesses were identified, the number of reported cases was doubled for each pathogen, and then multiplied by the proportion of infections caused by foodborne pathogens. It was estimated that 181,177 hospitalizations occurred each year, 60,854 of which were a

result of foodborne pathogens; of these foodborne cases, 60% were attributed to bacteria, 5% to parasites, and 34% to viruses.

It is believed that deaths due to foodborne transmission are also underestimated. In order to estimate the number of deaths, the same approach was used as was used to estimate the number of hospitalizations. Using this method, it was estimated that pathogens cause 2,718 deaths each year, and of these, 1,809 were due to foodborne transmission (Mead et al. 1999). Bacterial transmission attributed to 72% of deaths, parasites for 21%, and viruses for 7%. The most common pathogens were *Salmonella* spp. (31%), *Listeria* spp. (28%), *Toxoplasma* spp. (21%), noroviruses (7%), *Campylobacter* spp. (5%), and *E. coli* O157:H7 (3%).

In 1985, it was estimated that between 24 and 81 million illnesses could be attributed to foodborne pathogens (Archer and Kvenberg 1985). Two years later, it was estimated that foodborne pathogens only accounted for 6.5 million illnesses per year in the United States (Bennett et al. 1987). In 1989, it was estimated that 12.5 million illnesses are caused by foodborne pathogens yearly in the United States and Canada with 500 deaths (Todd 1989). In 1994, the estimates of illnesses caused by foodborne pathogens were at 33 million. Using data collected between 1996 and 1997, it was estimated that that rate of diarrhea was 1.4 episodes per person per year, and the rate of diarrheal illness lasting more than one day was 0.75 episodes per person per year (Mead et al. 1999). In addition, it was estimated that the rate of vomiting without diarrhea was 1.05 episodes per person per year. Estimates from rarer cases of gastroenteritis accompanied by respiratory symptoms resulted in 0.79 episodes per person per year. The rate of hospitalizations was estimated to be 4.7 per 1,000 people. Using the population of the United States in 1997 of 267.7 million,

these estimates result in approximately 211 million episodes each year. Of these 211 million cases, it is believed that 38.3 million can be attributed to known pathogens. Overall, it was estimated that cases of foodborne gastroenteritis in the United States is approximately 76 million per year, with more than 300,000 hospitalizations, and 4,300 deaths.

Mead et al. (1999) noted a number of assumptions used during this study. The first assumption was that the data reported was an underestimate of the total number of cases, and therefore multipliers were used based on previous data from the United States and other countries; however, it is stated that these multipliers may still result in underestimates, but may also result in overestimates as well. It is believed that the best multipliers were used in this study based on the information provided. The next assumption was the frequency of foodborne transmission of specific pathogens; data for this was extrapolated from previous studies with the addition of multipliers due to underestimates. The last assumption is the frequency of gastroenteritis and diarrheal illnesses in the United States (estimated 1.4 and 0.75 cases per person per year, respectively). These data were based on a random sample of 9,000 homes in the United States. Unfortunately, these illnesses were not specified as bacterial, parasitic, viral, or unknown. In addition, as the years progress, technology and knowledge has improved, resulting in more accurate estimates of rates of foodborne illness in the United States.

Through active and vigilant surveillance, the estimation of future foodborne illnesses will be more accurate. Improving technology and cost-effectiveness may also expand this surveillance to poorer areas lessening the degree of

underreporting. Lastly, identification of new causes of enteric illnesses and alerting the public to these dangers may also improve foodborne disease prevention efforts.

1.2 Viruses in Food

Viruses pose a threat to public health when present in food, and have gathered an increasing amount of attention in recent years as a viable source of foodborne infection. The most common pathogenic foodborne viruses are human enteric viruses. Foods that are more susceptible to viral contamination are those that do not require much processing, such as bivalve mollusks and fresh produce; these foods are generally infected with viruses in the primary production environment (CDC 2008). Other foods that have been implicated in foodborne outbreaks have been prepared, ready-to-eat foods contaminated by an infected food handler.

Viruses can be passed on to humans in a variety of different ways; however, the viruses generally associated with foodborne illnesses are primarily those that infect the gastrointestinal tract, which are usually found in feces and vomitus. In a report issued by the Council for Agricultural Science and Technology, human enteric viruses were ranked as the fifth and sixth most common causes of foodborne illness (CAST 1994). In addition, these viruses can be found on infected food handlers, as well as in animals. Another study reported that viral gastroenteritis was the most common foodborne illness in Minnesota from 1984 – 1991 with many of the outbreaks a result of infected food handlers (Hedberg and Osterholm 1993). Large-scale viral gastroenteritis outbreaks are generally a result of a combination of factors. The most common cause of foodborne viral gastroenteritis worldwide are noroviruses (NoV), hepatitis A viruses (HAV), and other small round-structured gastrointestinal viruses (Cliver et al. 1992). Foodborne outbreaks have also been attributed to

parvoviruses and astroviruses; less common foodborne viruses include enteroviruses, caliciviruses, aichi viruses (AiV), sapovirus, and coronaviruses. In addition, rotaviruses (HRV), some adenoviruses, and hepatitis E viruses (HEV) have been associated with waterborne outbreaks, especially in developing countries (Koopmans and Duizer 2004). NoV and HAV are extremely contagious, and person-to-person spread is the most common transmission route (CDC 2008). This can make it difficult to pinpoint the source of an outbreak of these viruses.

These viruses are commonly ingested via the fecal-oral route, and have been found to persist in the environment even in the presence of harsh conditions (pH, drying, radiation, etc). Based on the symptoms of infection, these viruses are generally placed into three groups: gastroenteritis viruses (NoV, HRV, HEV, astroviruses, AiV, adenoviruses, and sapoviruses), enterically transmitted hepatitis (HAV and HEV), with the third group infecting the intestine, and later causing illness after migrating to a different organ (enteroviruses) (CDC 2008). Common symptoms of viral gastroenteritis include vomiting and watery diarrhea, with other symptoms including headache, fever, chills, and abdominal pain, although asymptomatic infections are also common (Estes and Atmar 2006). Symptoms generally arise within 4 to 48 hours after exposure to the virus, and can last from 1 to 10 days.

The differences in morphology, infectivity, epidemiology, and persistence between bacteria and viruses cause many problems in risk management. Viruses are much smaller than bacteria (0.02 μm – 0.4 μm versus 0.5 μm – 5 μm , respectively). Most of the foodborne viruses described above are present in the environment and are able to withstand mild food processing procedures that are generally used to control bacterial growth. These viruses have small, single-stranded positive-sense RNA

genomes that are non-enveloped, although coronaviruses have an envelope; however, rotaviruses have double-stranded RNA genomes and adenoviruses and parvoviruses are DNA viruses. In addition, viruses need to enter cells in order to be able to replicate; however, foodborne viruses do not cause the deterioration of the food product or an alteration of the food properties which makes the viruses difficult to detect. Unlike bacteria, viruses only require a small amount of viral particles to cause infection; these particles are shed in high numbers in the feces of an infected person.

Foodborne viruses have recently come into the spotlight due to multiple factors. One main factor is that viruses act differently than bacteria, and most techniques for controlling foodborne outbreaks are effective against bacteria. Current control measures for viruses have not been widely tested, and therefore data are lacking on their efficacy toward viruses, as well as on actual control of viral contamination and spread (CDC 2008).

Recent studies have shown that viral infections due to food are common in many parts of the world, but are not necessarily reported as often as they should. This is due to the fact that the analytical and diagnostic tools for these foodborne viruses are not available in many countries. Current research is examining new approaches to identification of these viruses, including updating detection methodologies in food and clinical samples (CDC 2008). Research in recent years has shown an improvement in enteric virus detection in contaminated foods that are used in many countries; however, this information and technology are not available everywhere, causing some discord in methods of identification.

Improved methodologies will allow for the increasing accuracy of assessing the burden of foodborne disease linked to viruses, as well as improving

strategies for the prevention and control of viral contamination in foods and the associated risks. Currently, the most important factor in safe food handling practices is good general hygiene of both the food and the food handlers alike.

1.3 Routes of Transmission

The most common route of viral foodborne illness is the fecal-oral route, in that humans become infected after ingesting viruses present in fecally contaminated foods. Following ingestion, these viruses enter the gastrointestinal tract, survive the harsh acidic conditions in the gut, and initiate an infection. Viruses are concentrated in high concentrations in feces with some estimates suggesting up to 10^7 infectious virus particles per gram of stool (CDC 2008). These high levels allow for easy transmission upon contact. Another factor that enhances the infectivity of foodborne viruses is the ability to withstand harsh environmental conditions outside of the host, such as acid, heat, drying, pressure, disinfectants, and ultraviolet radiation. These factors greatly increase the risk of viral contamination of foods, especially those that do not encounter a great deal of processing prior to consumption. In addition, some animal viruses are able to infect human hosts. These viruses can enter into the food chain with animal products or with foods that have been contaminated with virus-infected animal manures. Once these viruses enter into the human population, person-to-person transmission is highly likely, such as HEV, H5N1, and SARS (CDC 2008). In most outbreaks, a combination of several transmission routes is often to blame. An example of this is the introduction of a virus into a susceptible food by contaminated water, which is then spread through the population via person-to-person contact or through the contaminated environment itself. The most common routes of transmission are described below.

The most commonly feared mode of transmission of viruses to food is human sewage and feces. A study conducted by Cadilhac and Roudot-Thoraval (1996) showed that HAV infections were prominent in sewage treatment workers in France. This brought about the suspicion that the current sewage treatment practices in place were not effectively removing or inactivating viruses. This is a major concern with bivalve mollusk consumption, as well as pre-harvest contamination of fresh produce. These contaminated sources could be applied via virus-containing irrigation waters, washing, and in fertilizers. Additional studies conducted in Europe, Japan, and the United States have shown that samples taken from sewage treatment plants tested positive for human enteric viruses at the end of the treatment procedures (ven de Berg et al. 2005, Ueki et al. 2005, Gregory et al. 2006). A distinct concern with sewage-related contamination is the possibility of infecting foods with multiple viruses (CDC 2008). This could result in a potential outbreak of multiple strains of viruses; with multiple virus strains (usually the same genus) replicating inside of a host cell, new strains of the virus can develop (Le Guyader et al. 2006, Symes et al. 2007). The introduction of these new viral strains within the host increases the difficulty of being able to pinpoint the source of the outbreak.

Another route of transmission is that of infected food handlers. If a person has an enteric virus infection, viruses can be detected in the stool of the infected individual in levels up to 10^7 virus particles per gram. In addition, these infected individuals may start shedding these virus particles as soon as 12 hours following exposure and can continue up to several weeks, depending on the viral infection (Rockx et al. 2002). These data suggest that an individual may have an asymptomatic infection at the beginning, and therefore not realize their infectiveness. Asymptomatic

infections are a common occurrence. Studies conducted in the Netherlands have shown that enteric virus shedding was present in 5.2% of control subjects (individuals without gastrointestinal complaints) and in 19% of individuals without gastrointestinal illness involved in an outbreak (de Wit et al. 2001, Vinje et al. 1997). Food handlers that are shedding viruses in their feces can inadvertently contaminate foods by not practicing proper personal hygiene; these viruses can be transmitted to foods and surfaces used for food preparation from contaminated hands (Bidawid et al. 2000, 2004). The contaminated surfaces used for preparation can also contaminate foods. Virus contamination can occur almost anywhere in the farm to fork continuity. For example, produce is handled by human hands during harvest, packing, distribution, and in the home. The persistence of viruses in a contaminated environment, their resistance to cleaning, and disinfection are all factors that may contribute to this mode of transmission (CDC 2008).

The third mode of transmission is animal viruses infecting human hosts, called zoonotic transmission. This can be directly seen in bivalve mollusks; when an oyster is infected with a human enteric virus that can cause an infection to a human host, the oyster would be a component in the route of infection. Likewise, if the oyster were to become infected with an animal virus, such as HEV from pig feces, and is consumed by a human, the mode of transmission is now zoonotic (CDC 2008). This can also be seen in infected meats and animal products. Studies have shown that HEV can be transmitted through raw meat, the liver of deer and wild boar, and in pig meat, organs, and feces (Tei et al. 2003, Takahashi et al. 2004).

1.4 Sources of Contamination

Knowing the routes of transmission of foodborne viruses to humans is the key to prevention strategies. Although more than one route of transmission may lead to the contamination of a food source, transmission routes are not necessarily of equal significance when considering the different food/virus combinations (CDC 2008).

This will also differ between countries with different standards of health and living conditions. For example, developing countries do not have clean water on a regular basis, and therefore may use non-potable water as irrigation water, or to clean/prepare foods. This results in higher levels of waterborne HRV in these countries.

Contamination of fresh produce is also of worldwide concern. Fresh produce can become contaminated by an infected food-handler, or with contaminated irrigation water; however, there is little data to supplement the transmission of foodborne viruses via contaminated irrigation water. Post-harvest conditions can also cause foodborne illnesses through contaminated preparation surfaces and infected food handlers.

Contamination of fresh produce through infected food handlers and contaminated irrigation water are current worldwide concerns. This is because fresh produce is currently grown on a large scale in many countries and is transported globally (CDC 2008). Many studies have been conducted on viral outbreaks on fresh raspberries and green onions, as well as leafy greens (LeGuyader et al. 2004, Amon et al. 2005, CDC 2003). This contamination can occur at both pre- and post-harvest, as described above. It was also found that in the case of green onions, some viral particles that may be present in irrigation water can be taken in while in the growth phase (Chancellor et al. 2006); however, these data are limited. Contaminated drinking water is of great concern when dealing with fresh produce. To make matters

worse, there is not a set standard of guidelines between countries on the quality of irrigation waters that may be used on crops. In some cases, the irrigation water used on fresh produce has been found to be contaminated with human waste (CDC 2008). In 2006, it was estimated that nearly 20 million hectares of farmland were being irrigated with contaminated water, increasing the risk of a foodborne infection (Hamilton et al. 2006).

As a society, we have become accustomed to having a year-long supply of fresh produce, increasing the demand for international trade. In 2006, it was estimated that nearly 9 million tons of lettuce and chicory were produced in developed countries, and another 14 million tons of lettuce and chicory were produced in developing countries (CDC 2008). Spinach production has also increased globally. In fact, leafy greens have developed high demand on an international scale as a result of increasing health and nutrition awareness. Between 1995 and 2005, the demand for lettuce increased by 50%, and the demand for spinach increased by 80% (CDC 2008). Berries, although not as popular as leafy greens, are also in high demand worldwide; in 2005, it was estimated that 500,000 tons of strawberries and 120,000 tons of raspberries were exported. Many of these berries were frozen prior to export, but this does not eliminate the risk of foodborne infections. Foodborne outbreaks can result in a loss of market for some countries; for example, an outbreak in the United States linked to raspberries from Guatemala resulted in a revoking of trading rights (Calvin et al. 2004). A similar situation occurred with HAV-contaminated spring onions exported from Mexico into the United States in 2003. These incidents resulted in the development of Good Agricultural Practices, or GAPs, among producers and as a requirement for future opening of export markets (CDC 2008).

1.5 Good Agricultural Practices (GAPs)

While the majority of fresh produce consumed in the United States are clean and free of pathogenic microorganisms, recent foodborne illness outbreaks have resulted in the need for a more globally standardized method for food production. Good agricultural practices (GAPs) are a collection of principles to apply for on-farm production and post-production processes that result in safe and healthy food and non-food agricultural products, while taking into account economic, social, and environmental sustainability (Johnson et al. 2000). Four main points brought about the need for GAPs: (1) recent reoccurring outbreaks linked to consumption of fresh produce, (2) positive detection and recovery of human pathogens from random survey sampling of fresh produce, both domestic and imported, (3) recent reports from several studies suggesting the difficulties in decontamination of fresh produce, and (4) recent reports from several studies suggesting the potential for internalization of pathogens post-harvest (VanVranken et al. 2001). With the varying wide range of environments, crop management, and handling practices, a single approach to food safety is unlikely; however, using a set of guidelines for the reduction and prevention of viral contaminants, fresh produce production can become safer.

Contamination of fresh produce is a result of an external environmental source at some point from production to post-harvest (VanVranken et al. 2001). Alerting and educating these producers to the potential risks and good management practices will minimize the chances of contamination at every step from farm to fork. Once the produce is contaminated, it is difficult to remove the pathogen; therefore, it is much easier to prevent contamination at all steps than decontaminating the produce.

Water comes in contact with fresh produce at almost every step from farm to fork, so it is key to use potable water to minimize the risk of contamination. Fresh produce farmers are advised to familiarize themselves with the routes and handling of water sources, and to identify any potential sources of contamination that can be avoided. Under the GAP guidelines, the fields used for fresh produce should be divided into agricultural and post-harvest sections (VanVranken et al. 2001). The best quality water is used in the post-harvest sector due to the high water-to-produce contact. In addition to using potable water, GAPs suggest the addition of antimicrobials to the water to enhance the water quality. The agricultural sector, on the other hand, is more lenient in the quality of irrigation water used. Many farms will use reclaimed or recirculated water in areas where water is a scarce commodity.

Farmers are advised to minimize the contact of irrigation water with fresh produce, favoring drip or furrow irrigation to spray irrigation (VanVranken et al. 2001). Studies suggest that avoiding overhead irrigation five days prior to harvest will greatly reduce residual pathogens on plant surfaces. In the case of using well water, construction and location of the well are important. The well should be on an elevated surface that will encourage drainage away from the water source. The well should also be protected from contaminants that may be near the well, including protection from run-off and flooding. Prevention of water contamination is the basic part of any food safety plan, and should remain a priority for farmers. The water source must also be protected from animal contamination, either from animal waste or from animals grazing near the water source; these animals can be both livestock and wild.

The water used to deliver chemicals and other amendments to plants must also be of post-harvest quality (VanVranken et al. 2001). This water is used to deliver

pesticides, growth regulators, nutrients, etc. to fresh produce, and may come into contact with the edible portion of the plant. This is an example of a preventative measure. The post-harvest water must be high quality as well. Post-harvest water is used for transferring produce to field bins or trailers, gently floating easily-bruised product from the field bins, removing soil and debris, and removing initial field heat. Wash water used in post-harvest can be applied by submersion or by spraying. The submersion method is more likely to spread contamination directly, whereas spraying can spread contamination by direct contact or by creating contaminated aerosolized particles. To prevent this, the wash water must be of high quality. In addition, it is suggested that fresh produce undergoes multiple washes to reduce the amount of microorganisms on the surface of the product, as each wash can reduce the amount of microorganisms by 10- to 200-fold.

In addition to water, it is important to monitor the use of manure and municipal biosolids. If these biosolids are properly composted, microbial pathogens are of little concern (Foess and Fredericks 1995); however, the risk of viral contaminants is still high. To avoid potential catastrophe, produce growers are advised to maximize the time between application of manure to production areas and harvest. It is also important to keep animals away from the growing fields as much as possible; fences and buffer zones should be used to reduce the presence of wild animals.

Food-handler hygiene is also important. Training programs have been implemented to educate food handlers of the importance of personal hygiene and proper hand-washing techniques. Workers that are ill or may be ill are required to be reassigned to activities that do not involve food or a food surface; if this is not a

possibility, the food-handler should be sent home until the signs of the illness pass. In addition to food-handler sanitation, field and harvest sanitation is also important. All surfaces and tools that come into contact with fresh produce must be treated as food contact surfaces. Included in this is transportation and distribution of produce. Transportation and distribution pose the possibility of cross-contamination. To reduce this risk, GAPs suggest separating dry and wet products, as well as constructing a water-repellant barrier between mixed loads.

When a product is found to be contaminated, the samples taken for testing should come from random areas and need to be representative of the processing area (VanVranken et al. 2001). When retrieving the samples, it is important not to cross-contaminate the product; cross-contamination will ruin the sample and make it impossible to determine where the contamination came from. In addition, the samples should be tested as soon as possible after sampling to get the most accurate reading.

1.6 Climate Variability and Its Impact on Pathogenic Microorganisms

Environmental health-related areas affected by weather and climate-related conditions are waterborne diseases that may be transmitted via drinking water and recreational waters, foodborne diseases caused by contaminated water, and marine-associated issues, such as harmful algal blooms (HABs) (Rose et al. 2001). These HABs can accumulate in filter-feeding shellfish, which can, in turn, harm humans consuming these shellfish. In the United States, it is estimated that 9 million cases of waterborne disease occur each year (Bennett et al. 1987); however, this is only an estimate; many cases of gastroenteritis go unreported. Most cases of gastroenteritis are self-limiting and short-lived, although some cases (i.e. hepatitis) can result in extended illness. In addition, some waterborne pathogens have been found to

be associated with hepatic, lymphatic, neurologic, and endocrinologic diseases, as well as possibly increasing the risk for certain cancers. Exposure to waterborne pathogens can occur via ingestion, inhalation, and even absorption through the skin, and can affect both freshwater and marine waters alike. Ecologic factors such as overfishing, bottom trawling, introduced species, altered freshwater levels, increased nutrients, UV radiation, and abrupt climate changes all pose a threat to pathogenic microbial contamination (Hader et al. 1995). For example, high levels of precipitation can decrease the effectiveness of sewage disposal, and may cause runoff into drinking water, freshwater bodies, or coastal waters. In addition, high levels of rainfall can cause runoff from agricultural areas with livestock to growing fields and drinking water reserves.

1.7 Waterborne Diseases

Drinking water contamination is not as large of a problem in developed countries as it is in developing countries. In the United States, about 10 to 15 cases due to contaminated drinking water are reported each year with more that may potentially go unreported (Payment et al. 1997). Between 1971 and 1996, there were 674 reported waterborne outbreaks in the United States (Levy et al. 1998). In 1993 and 1994, it was estimated that 405,366 people were infected in the United States due to contaminated drinking water. This contamination can come from a number of different factors, such as contaminated water source, transmission of the contaminant to the water intake/well, ineffective treatments to remove contaminants from the water, and finally exposure to the contaminant (Rose et al. 2001). More than 100 different pathogenic microorganisms have been found in contaminated water.

In 1993, there was an outbreak of *Cryptosporidium parvum* in Milwaukee, Wisconsin that was found in drinking water, infecting 403000 individuals and resulting in 54 deaths (Ford 1999). This protozoan is shed in high numbers in feces, and thrives in mammalian intestines. The water supply for Milwaukee originates in Lake Michigan, and undergoes filtration and chlorination prior to dispersal; however, in this particular instance, a period of heavy rainfall and runoff decreased the quality of the water source, and therefore decreased the effectiveness of the purification system. Waterborne outbreaks of cryptosporidiosis have been reported around the world, generally following periods of heavy rainfall. Shortly after the Milwaukee outbreak, several cases of *C. parvum* were reported among patients with AIDS in Las Vegas, Nevada (Goldstein et al. 1996). *C. parvum* is the most prevalent parasitic waterborne infection, with *Giardia lamblia* the second most prevalent in the United States. In a study conducted by LeChevallier et al. (1991), it was found that in 66 tested water treatment plants in 14 states, 87% were positive for the presence of *Cryptosporidium*, and 81% were positive for the presence of *Giardia*; however, these plants also met the turbidity requirements of the time and were not penalized for these results.

Human wastes in marine and freshwater environments may be due to insufficient waste removal systems, inadequate disinfection of sewage effluents, outfalls, and storm water (Rose et al. 2001). Combined sewer systems, which carry both storm water and sanitary wastewater through the treatment plant, are a continuing threat to water quality and public health. When precipitation is high, the volumes traveling through the treatment plants can exceed the capacity of the pipes; the system then defaults to discharging the excess water into surface water bodies, which then

transfers any pathogens present into the water (Perciasepe 1998). It is estimated that nearly half of the water-related contamination is due to storm-water runoff.

Waterborne pathogens can also be found in recreational waters that are used for swimming, fishing, boating, etc. (Dufour 1984). Studies have found that pathogens in recreational water sources have led to problems with the eyes, ears, nose, skin, respiratory tract, and gastrointestinal tract. These bodies of water can become contaminated via urban runoff, industrial pollution, storm waters, human and animal wastes, and native sources (i.e. red tides). In 1996, 3700 beaches were closed in the United States because of the detection of high levels of bacteria; the majority of these beaches were located near storm drains (Haile et al. 1999). Freshwater systems are at risk for fecal-associated microbes and free-living parasites, such as *Naegleria fowleri*, which causes meningoencephalitis. Studies of marine systems have demonstrated a linear relationship between the incidence of gastroenteritis among swimmers and the levels of marine enterococci and *E. coli* (Cabelli et al. 1982). This same study also found that gastrointestinal symptoms decreased with increasing distance from municipal wastewater sources.

Approximately 50% of the population of the United States depends on groundwater from wells for drinking water, 23 million of which own private wells; the remaining population uses surface waters, such as lakes, rivers, and reservoirs (EPA 1997). In 1974, the Safe Drinking Water Act was enacted, establishing requirements protecting the quality of drinking water. The regulations under this act account for more than 80 contaminants, including organic chemicals, radionucleotides, and microorganisms. General disinfectant practices require the addition of chlorine to the water to reduce the number of microorganisms; however, some private well users rely

on untreated well water that is generally found in shallow wells; studies have shown that pathogens can pass through filtration and disinfection systems in place (Rose 1997). In 1996, the Safe Drinking Water Act was improved to include groundwater protection in these areas, and in 1998 the Clean Water Action Plan was implemented focusing on watershed protection. These improved regulations focused on pollution protection of watersheds.

Unfortunately, the current system does not detect contamination events until after outbreaks have occurred; this is because most cases are not reported to the health department (Rose et al. 2001). Continuous assessment of water quality and potential risks to water sources are key for prevention of contamination and outbreak events. In addition, wastewater management can be improved as well. Increasing populations burden the current waste treatment programs in place. Research has shown that combined sewer systems and separate sanitary sewers have an average flow of 173 gallons per capita per day, which is above the EPA maximum flow guidance of 100 gallons per capita per day (Parker et al. 1983). Increased precipitation can increase this load, decreasing the efficiency of treatment systems. Watershed protection is also important, as the quality of the watershed directly impacts source water and finished water quality, as well as recreational sites and coastal waters (Rose et al. 2001). Increasing the efficiency in which agricultural and city wastes are captured and treated could potentially reduce the amount of runoff of nutrients, toxic chemicals, trace elements, and pathogenic microorganisms flowing into reservoirs, groundwater, lakes, rivers, estuaries, and coastal waters. It has been found that storms cause transport of more than 60% of the annual load of

contamination in urban areas. The reduction of these effluents would improve the quality of water systems.

1.8 Foodborne Diseases

In the United States, foodborne illnesses are estimated to be 76 million cases per year with 325,000 cases resulting in hospitalization and 5,000 deaths (Mead et al. 1999). It is believed that this number is increasing as the years progress due to an increasing susceptible population, including the elderly and immunocompromised, as well as increasing worldwide trade. In particular, the water-food connection is apparent, as microbial agents in water can contaminate food (Rose et al. 2001). Fresh produce is increasingly becoming a factor in foodborne outbreaks, which may be a result of changing diets worldwide.

Changing weather conditions have been shown to contribute to the contamination of coastal waters and shellfish-associated disease. Fish and shellfish from contaminated waters have also been sources of foodborne outbreaks in the United States. Shellfish-associated outbreaks in the United States have included pathogens such as *Salmonella* Typhi, *Campylobacter* spp., *Vibrio* spp., *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* (Jaykus, Hemard, and Sobsey 1994). *Vibrio* spp. have been found to be strongly associated with weather factors, particularly temperature, and are therefore generally seasonal (Motes et al. 1998). *V. vulnificus* is naturally occurring in temperate estuaries and is rarely recovered in winter months; however, it is found year-round in more subtropical regions (Rose et al. 2001). *V. vulnificus* can cause mortality when associated with consumption of contaminated shellfish and thrives under moderate salinity (Colwell 1996). *V. cholerae* is

responsible for outbreaks of cholera around the world and has been found in plankton and fish in ponds and coastal waters in pandemic areas.

Human sewage contains viruses that can contaminate raw shellfish. These viruses have been found to be abundant in marine systems and even outlive their bacterial counterparts (Birch and Gust 1989, McDonnell et al. 1997). Studies have found viruses in sewage and storm waters in concentrations up to 3,000 particles/L, and after sewage treatment, as many as 40% of viruses survive (Seyfried et al. 1984). In this same study, enteroviruses were found in 40% of recreational waters that were considered safe by fecal coliform counts. Viral outbreaks have also been associated with baked, broiled, steamed, or fried shellfish (Lipp and Rose 1997). In the 1960s, hepatitis A was the most predominant seafood-related disease reported; today, Gastroenteritis is the most prevalent, generally caused by caliciviruses (LeGuyader et al. 1996).

Cyclospora is a protozoan species implicated as an etiologic agent of prolonged watery diarrhea, fatigue, and anorexia in humans (Rose et al. 2001). In 1996, 1,465 reported cases of cyclosporiasis were reported in 20 states in the United States, and in 1997, several more cases were reported that were associated with raspberries, lettuce, and basil. Most of these cases occurred in the late spring and early summer months (Rose et al. 2001).

Runoff from high levels of rainfall contributes to the contamination of coastal waters and shellfish-harvesting areas (Rose et al. 2001). A study conducted by Lipp et al. (2001) found that the concentrations of fecal indicator organisms during the winter of the high-precipitation El Niño of 1997-1998 were significantly higher than

the concentrations found the previous year. Infectious enteroviruses were found in 75% of the samples taken, as compared to none found the previous year.

1.9 Role of Climate

Climatic events, in particular precipitation and temperature changes, have been found to facilitate contamination of sources of drinking water and food, as well as creating coastal water issues. Worldwide studies have shown a relationship between diarrheal diseases and climate; for example, increased temperatures in Peru during the 1997-1998 El Niño season resulted in a doubling of the number of children hospitalized with diarrhea (Checkley et al. 2000). The majority of these hospitalizations were due to cyclosporiasis; the *C. cayetanensis* spores matured quickly in the warmer temperatures.

Data on drinking water outbreaks in the United States from 1948 to 1994 including all possible infectious agents demonstrated a seasonal trend, a spatial clustering in watersheds, and association with precipitation levels (Rose et al. 2000). It is believed that watersheds located near human sewage and animal wastes are at a higher risk for fecal contamination with higher levels of precipitation. In September 1999, the largest waterborne outbreak of *E. coli* O157:H7 in the United States occurred in New York State at a fairground following heavy rains; it was found that the well water was contaminated (Rose et al. 2001).

Climatic and environmental factors, linked to rapid monitoring tools are in the process of being developed as an early-warning system for potential outbreaks. The goal of these systems is to advise the public and prevent outbreaks by including boil-water orders, shellfish-bed closings, and temporary bans on seafood consumption (Rose et al. 2001); however, in order to put systems such as these in place, additional

research must be implemented to determine the relationships among temperature, sea-level rise, climatic factors, and the ecology of disease agents. In addition, tools need to be in place to monitor any changes in physical, chemical, and biological parameters. The data collected would then be entered into a database, and significant changes would be noticed more readily to prevent any outbreaks that may occur. Waterborne diseases remain a major public health problem in the United States, which are generally caused by climatic factors. Therefore, improved surveillance, watershed and water-source protection, and educational programs are all necessary to understand and prevent outbreaks.

1.10 Virus Survivability in the Environment

Viruses cannot replicate outside of a host cell, and therefore cannot proliferate during processing, transport, or storage. Unfortunately, most foodborne and waterborne viruses are resistant to heat, disinfection, and pH extremes. This viral characteristic allows for low levels of foodborne viruses to be present on a food source which will still induce an infection. Past studies have demonstrated the transmission of human rhinoviruses (HRV) found in aerosols (generated via vomiting) to food products (Sattar et al. 1984). This study showed that the HRV in the aerosol particles was able to survive in the air for up to 9 days at 20°C. Similar studies have found that viruses may survive for up to 60 days on many materials found in the kitchen such as paper, cotton cloth, aluminum, china, glazed tile, latex, plastics, and polystyrene (Abad et al. 1994, Nauheim et al. 1990).

Most foodborne viruses can survive for prolonged periods of time in a pH range from 3 – 10. Enteroviruses prefer high relative humidity, while HAV and HRV favor lower relative humidities (Mbithi et al. 1991, Sattar et al. 1988). In

contaminated water, polioviruses and rotaviruses may survive in temperatures as low as 4°C (Biziagos et al. 1988). Another study conducted by Croci et al. (2002) showed that some enteric viruses can survive on fresh produce for extended periods of time, sometimes outlasting the produce itself. HAV and HRV are more resistant to inactivation than enteric adenovirus and poliovirus (Croci et al. 2002). These data present the issue of developing virus-specific methods of food decontamination.

Differences have also been found in virus survivability under different processing and substrate conditions. Washing fresh produce with water and with a disinfecting agent will only remove 1 – 2 log₁₀ of the amount of virus on the food (Papafragkou et al. 2006). In addition, refrigeration and freezing of foods have little to no effect on viral inactivation, and may even help preserve the virus particles, especially in freezing conditions. This suggests that precautions must be taken in preharvest conditions in foods that undergo little processing. Of course, foodborne transmission is not just a result of adverse environmental conditions; if foods are contaminated at the end of processing, or prior to consumption, it is not necessary for the viruses to survive an extended period of time outside of the host. These viruses just need to be able to withstand the acidity of the gut to cause gastrointestinal infection.

Prevention of this end of the food-chain contamination is key, and therefore decontamination of hands and surfaces used to prepare this food is of utmost importance. Hand sanitizers remove most viruses, but no agent completely removes or inactivates enteric viruses from hands (Steinmann 2004). In a study conducted by Sattar et al. (2002), it was found that some hand sanitizers only resulted in a 1 – 2 log₁₀ drop in the virus titer. The most effective way to remove viruses from the hands

is proper hand-washing; alcohol-based hand sanitizers are not nearly as effective as soap and running water (Bidawid et al. 2000). Decontamination of surfaces is also highly important. The survival and transferability of enteric viruses on surfaces is dependent upon multiple factors, such as temperature, relative humidity, type of surface, and virus (CDC 2008). Enteric viruses survive on surfaces at room temperature and moisture for days to weeks, and were able to be transferred between surfaces, hands, and foods with ease (Papafragkou et al. 2006). Frequent cleaning and decontamination of food preparation surfaces is therefore important in the control and removal of foodborne viruses.

In many cases, HAV and HRV resist inactivation by commonly used preservation and processing methods, whereas enteric adenovirus and poliovirus are much more susceptible to these methods. In fact, studies have shown that HAV has high resistance to inactivation by heat, desiccation, pH extremes, and ionizing radiation (Koopmans and Duizer 2004), whereas adenoviruses have shown the greatest resistance to ultraviolet radiation (Thurston-Enriquez et al. 2003), and polioviruses have shown the greatest resistance to high pressure processing (Kingsley et al. 2002). Of course, these studies were conducted under laboratory conditions, and cannot explicitly answer for what we see in nature.

1.11 Reported Foodborne Illness Worldwide

There are many gaps in the current knowledge of the incidence of foodborne illness worldwide. This is mainly due to the fact that person-to-person spread of foodborne viral illnesses is quite common, causing difficulties when trying to pinpoint the source of the outbreak. In many cases, such as those involving noroviruses (NoV), epidemiological studies are not available. In fact, most

epidemiological studies severely underestimate the occurrence of viral foodborne illnesses. For example, in a study conducted by Wheeler et al. (1999), it was found that in the United Kingdom, only one out of 1,562 cases of NoV was pinpointed to a specific food source. The researchers blamed these findings on three main factors: NoV is a self-limiting infection and medical treatment is not generally sought, clinical diagnostic methods are virtually nonexistent, and an overall lack of all-encompassing viral foodborne outbreak research. A few specific population-based studies have been conducted estimating the fractions of illnesses that are foodborne, which is shown below: (Source CDC 2008) (Table 1).

These studies only provide rough estimates based on the information that was available at the time, and use data from differing sources. Viruses are indeed an important source of foodborne illness. Estimates have placed the proportion of viral foodborne illnesses in a range from 13,000 to 30,000 per million people (Mead et al. 1999). Unfortunately, little information has been collected pertaining to the rates of hospitalization and death due to foodborne viral agents. In the same study by Mead et al. (1999), it was suggested that two-thirds of all foodborne illnesses, one-third of hospitalizations, and seven percent of deaths from foodborne disease were viral in the United States.

Between 1994 and 2005, the National Institute for Public Health and the Environment in the Netherlands began a systematic surveillance of gastroenteritis outbreaks that were caused by viruses (Svraka et al. 2007). Beginning in 1994, viral gastroenteritis outbreaks were reported based on fecal samples; cases were interviewed on living conditions, possible transmission routes, time of onset of symptoms, and hospitalization. In 1994, the only method of viral detection for

gastroenteritis was electron microscopy; however, in 1996, RT-PCR for noroviruses was developed, becoming the new method of routine diagnostic measures (Vinje and Koopmans 1996). If the samples tested negative for noroviruses, the samples were assayed for the presence of sapoviruses, astroviruses, adenoviruses, and rotaviruses; a total of 373 samples were tested. As the years progressed, noroviruses became better understood, and RT-PCR detection methods were expanded in 2004 to distinguish between genotypes I and II. Detection methodologies for sapoviruses were developed in 1999, while ELISAs were used for the detection for rotaviruses, adenoviruses, and astroviruses until 2002. In 2002, PCR methods were developed for rotaviruses in groups A, B, and C using primers that target the RNA-dependent RNA polymerase of these viruses. In addition, previous unexplained viral outbreak samples were retrospectively examined (Svraka et al. 2007). The outbreaks occurred before 2005 and were tested for norovirus group I and II. Samples that tested negative for noroviruses prior to 2003 were retested for rotaviruses, adenoviruses, and astroviruses. Samples that tested positive for viral RNA were confirmed by sequencing using fluorescence-labeled dideoxynucleotide technology.

At the end of this study, 6,707 stool samples from 941 cases were collected, and 76.4% of these cases tested positive for norovirus, 4.4% were positive for rotavirus, 0.7% were positive for adenovirus, and 0.5% were positive for astrovirus (Svraka et al. 2007). Retesting of the 140 unexplained cases found 16 to be caused by norovirus, 5 by rotavirus, 2 by adenovirus, and 2 by multiple pathogens. Most of the norovirus related outbreaks occurred between October and March, with the peak in December. The season for rotaviruses occurred between December and April, and outbreaks of adenoviruses and astroviruses generally occurred in March. It was also

found that the number of reported outbreaks, especially those caused by noroviruses and rotaviruses, increased over time. It is believed that this may be due to the emergence of more virulent strains, strains entering the Netherlands that were not native to the region, or just a general increase in general knowledge concerning the viruses and their symptoms (Lopman et al. 2004). At the time this paper was published, aichi viruses and sapoviruses were not found to be causative agents of gastroenteritis outbreaks in the Netherlands, and only one AiV outbreak had been reported in Germany and three in France (Svraka et al. 2007). This study showed that detection methods are improving with improving technology and understanding of pathogens, and are therefore becoming more sensitive and have a wider scope than those used previously.

1.12 Leafy Greens Pre-Harvest

The World Health Organization defines leafy greens as encompassing all vegetables and herbs of a leafy nature in which the leaf and core are typically eaten raw. Since 1980, the worldwide production of leafy greens has increased by nearly 94% (EU 2007). Consumption of fresh produce (both fruits and vegetables) increased by an average of 4.5% per year between 1990 and 2004. In 2006, the major producers of lettuce and chicory were China (50%) and the United States (20%) with China also producing 84% of the world's spinach (FAOSTAT 2008). Many of these leafy greens come in packaged salad mixes. Increased consumption is due to recent healthy eating campaigns that suggest that daily consumption of fresh produce can help prevent major diseases such as cardiovascular diseases and some cancers. Studies have shown that all fruits and vegetables are able to contribute to this benefit with leafy greens leading the pack (Hung et al. 2004, Link and Potter 2004). It is estimated that the

production and consumption of leafy greens will continue to steadily increase worldwide.

Leafy greens naturally have microbiota living on the surface of the product, much of which is non-pathogenic; however, along the farm-to-fork continuum, there is risk for further, more pathogenic contamination. Beuchat (2006) found that leafy greens hold some of the highest risk for transmission of foodborne pathogens; however, data directly linking foodborne illness outbreaks to leafy greens is also limited by the fact that leafy greens are typically mixed in salads or with other foods that could also be a vector for foodborne illnesses. When these outbreaks occur, the source is typically referred to as “salad,” “green salad” or “coleslaw” so as to not wrongly identify the specific source of the outbreak (WHO/FAO 2008). It is assumed that the estimates of foodborne illness outbreaks are severely underestimated; most reports on the incidence of foodborne disease associated with leafy greens are based on outbreaks and not sporadic cases. In addition, when an outbreak is attributed to “salad,” it is difficult to trace the outbreak back to the source, thereby adding to the underestimations.

Reported outbreaks associated with leafy greens are generally widely distributed globally, with many consumers becoming ill. Between 1998 and 2005, outbreaks attributed to fresh produce as a result of leafy greens were 70% in the United States and 75% in Brazil (WHO/FAO 2008). Foodborne illnesses attributed to fresh produce account for 2.9% of the total foodborne illnesses recorded in the United States, and account for 4.8% of the outbreaks and 6.5% of the illnesses recorded worldwide (Lynch et al. 2006, Herman et al. 2008). These statistics were attributed to

the increasing consumption of leafy greens; however, increased consumption was not the only factor contributing to these increased reports of foodborne illness.

As previously stated, fresh produce has naturally occurring microbiota that is generally non-pathogenic, although contamination is quite possible. Contaminants can come from the growing field environment (water, soil, animals, etc.), farm equipment, and farm workers. The survival and proliferation of these contaminant microorganisms depend on a number of factors, such as the characteristics of the microorganism, the physiological state of the plant and its inherent resistance to microbial metabolic processes, the plant environment (pH, water activity, atmospheric composition), and the effects of any processing procedures (deRoever 1998). Unfortunately, worldwide data gaps are present due to availability of technology and resources.

Many of the recorded outbreaks due to fresh produce are a result of bacterial contaminants. Studies have reported levels of up to $8 \log_{10}$ MPN/g coliforms on fresh produce; however, some of these recorded coliforms may be part of the natural biota and cannot be specifically counted toward levels of foodborne pathogens. The bacterium used for detection of fecal contamination is *E. coli*. In a study conducted by Mukherjee et al. (2004), *E. coli* was found in 10.7% of field samples of leafy greens, with 22.4% of the contamination found in lettuce, 10.2% in cabbages, and 13.3% in bok choy. The average count of *E. coli* in this study was $3.1 \log_{10}$ MPN/g, and exposure to risk factors for fecal contamination was linked to higher recorded counts of *E. coli* contamination (e.g., crops fertilized with improperly composted manure) (Mukherjee et al. 2004, 2007). Bacterial contamination of foods that are not associated with an outbreak is generally found in small numbers compared

to the natural microbiota found on the surface of the produce; these bacteria are selected for and amplified for further testing (WHO/FAO 2008). In order for a product to be considered contaminated, 25 g of sample are tested for presence of the bacterium. Analysis for viral contamination is not as straightforward, many requiring DNA/RNA analysis for identification.

The increased focus on healthy eating and nutrition has caused a global expansion of fresh produce production, including larger crops and new crop production to supply the year-round demand. Cultivation practices differ between countries, but there are two general methods used: open field and protected cultivation systems (WHO/FAO 2008). The differences that come into play are size, location, productivity, target market, and crop needs. The protected cultivation method allows for year-round crop cultivation, thereby increasing overall yield and profit. These crops are not exposed to as many pests and potential contaminants as in the open field system, but require a higher level of inputs per hectare. Therefore, if contamination were to occur in a protected cultivation system, the chances of it spreading to nearby plants is increased. Some farmers also employ a soilless cultivation system (Gruda 2009). These soilless systems provide growers with shorter growing cycles and a high turnover. Crops grown in soilless systems generally have higher nutritional values due to highly regulated growing conditions; there is a universal requirement for safe water and hygiene control of the aquatic systems. There are also organic and conventional crops, which differ in the use of natural fertilizers and the avoidance of the use of chemicals in the control of contaminants.

Regardless of the method chosen to grow fresh produce, there is always the risk of contamination from both accidental and intentional inputs (Beuchat 2006,

Brackett 1999). The main sources of contamination are adverse weather conditions (e.g., flooding), topography of the growing fields, wildlife, livestock, human error, non-potable water, and soil amendments.

Contamination of fresh produce from animal sources (both wild and livestock) is of great concern. Contaminants from these sources include *Salmonella*, *E. coli*, *Shigella*, *Listeria monocytogenes*, norovirus, and HAV. Spread of contamination from an infected animal to a crop depends on the pathogen itself and the degree of interaction between the animals and the growing environment (Morris et al. 1994). Fresh produce can be contaminated by these sources through fecal matter, urine and hair from live animals, and carcasses in growing fields, which can be transferred directly or indirectly through environmental factors, such as water, insect vectors, contact with farming equipment, or by food handlers (Jay et al. 2007). The presence of *E. coli* O157 in cattle is a great concern to leafy greens production with an average of 3.3 log₁₀ cfu/g found in feces of infected animals (Berg et al. 2004). Other potentially harmful strains of *E. coli* have been found in deer, sheep, goats, horses, pigs, turkeys, and dogs (Doane et al. 2007). Noroviruses can infect humans, pigs, cattle, and mice, leading to the possibility of zoonotic transmission to fresh produce (Mattison et al. 2007). Indirect contamination of fresh produce is also a great concern. This indirect contamination can be a result of contaminated water, aerosols and dust from livestock production/feeding facilities, landfills, and wastewater treatment sites. The latter two sources may use wildlife as a vector, as these sites attract animals, which may go on to drink from and contaminate a water source that feeds to the farms (Nesse et al. 2005).

The topography and climate of the growing fields also play a role in the chances for contamination. The location of a fresh produce farm could determine the overall risk of potential foodborne contamination. Farms located downstream from heavily populated areas or areas that are industrialized are more prone to potential contamination. These farms must be wary of run-off water reaching the growing areas that could be contaminated, as well as wind that may carry contaminant dust particles. Studies have been conducted showing that dust is a prime vector for microbial contamination; *Salmonella* has been shown to survive up to 26 months on dust particles, and *E. coli* has been found to survive up to 10 months (Davies and Wray 1996, Varma et al. 2003); however, data has not been shown linking the contamination to foodborne outbreaks. Farms located in valleys are also at risk for contamination from higher elevation runoff. In a study conducted by Brown et al. (1992), it was found *Giardia* had been transported through runoff from higher elevations in remote areas in New Zealand.

Spastic weather conditions also affect the risks for contamination. Dry conditions increase the presence of dust, which may be contaminated and settle on leafy greens (Baertsch et al. 2007, Varma et al. 2003). Increased temperatures also potentially increase the rate of microbial growth, as well as influence the number of insect vectors that may also carry contaminants (Epstein 1995). Pathogenic microbial growth post-harvest can also result due to improper storage temperatures. Abrupt and unexpected changes in the weather may also affect the leafy greens' growth and physiological status, which may enhance their susceptibility to contamination (Dreux et al. 2007). Studies have also shown that increased UV light may decrease the number of human pathogens on leafy greens (Zaafrane et al. 2004).

Flooding of produce fields may cause contamination problems in some areas; microbial contamination of flood water can be a result of fecal waste, non-potable water, and contaminated soil. Flooding following drought conditions also increase the risk for contamination, as runoff is generally more severe (WHO/FAO 2008). Recent studies conducted in the United States have shown that nearly 50% of waterborne outbreaks are a result of prodigious amounts of rainfall (Curriero et al. 2001). Excessive rainfall also affects wells, which are set to optimally perform at a predetermined water level. If that water level increases, contaminants can enter the system (Charron et al. 2004).

Soil amendments can also introduce contaminants unto leafy greens. This is generally seen with organic fertilizers that are contaminated with bacterial, protozoan, and/or viral pathogens. Organic fertilizers are used in many parts of the world to provide additional nutrients to crops. Although these fertilizers are economically and ecologically necessary for the management of animal and human wastes, these soil amendments also provide excellent environments for pathogens. These contaminants can easily associate with leafy greens based on the type of manure, the frequency of application, and the time in between application and harvest (WHO/FAO 2008). Studies have shown that *E. coli* and *Salmonella* can survive in manure for up to two years, although survival depends upon many factors, including pH, temperature, fiber content, and oxygen content (Jiang et al. 2002). Additional studies have provided data showing the decrease of these pathogens in cattle fed a high fiber diet (Franz et al. 2005). Survival of these pathogens is decreased in manure-amended soil, generally because of greater temperature fluctuations and lower levels of available nutrients (Fremaux et al. 2008); however, leafy greens grown in

manure-amended soils are also at risk for pathogen association. The pathogens present in the soil at the time of planting may be able to colonize the plant, although studies have been inconclusive as to whether the amount of colonized pathogens present are at levels that are detectable, and internalization of these pathogens has not been found (Franz et al. 2005, Jablasone et al. 2005). Manure has also been arraigned in the spread of foodborne viruses to leafy greens, with partial genetic sequences of noroviruses found in cattle and pigs (Mattison et al. 2007). Studies have shown that enteric viruses may be more persistent in manure and manure-amended soils than bacterial pathogens (Gessel et al. 2004). Additional studies have found that viruses can survive in semi-liquid cattle manure from one week (herpesvirus) to over 6 months (rotavirus) (Pesaro et al. 1995).

The recycling of human biosolids for agricultural use is also of concern with fresh produce; human biosolids are generally used as fertilizers and soil amendments. The use of human biosolids for agricultural purposes varies between regions. In the United Kingdom, biosolids must be applied to crops between 12 and 30 months prior to growing, for vegetables meant to be cooked or eaten raw, respectively (ADAS 2001). These regulations further state that if the biosolids are treated to reduce bacterial and viral pathogens, 10 months may be deducted between biosolids application and growing of leafy greens. The use of human biosolids over animal wastes for leafy greens production raises the concern of an increased level of human pathogens introduced to growing areas, which could ultimately lead to an outbreak. The presence of human viruses, such as HAV and noroviruses, in human biosolids creates additional risks for leafy greens production. Studies have shown that HAV is highly resistant to desiccation of feces and is considered more thermally

resistant than *E. coli* (McCaustland et al. 1982). Additional studies have shown that common methodologies used for sewage treatment removes most harmful bacteria, but does little to inactivate pathogenic viruses, and increased thermal treatments are required to inactivate thermostable viruses (van de Berg et al. 2005, Spillman et al. 1987).

Illnesses caused by waterborne pathogens occur all over the world. The use of non-potable water on crops increases this risk of spreading contamination. Many waterborne outbreaks have been attributed to bacteria (*Salmonella enterica*, *E. coli*, *Campylobacter* spp.), intestinal helminths, amoebae, and protozoa (*Giardia intestinalis*, *Cryptosporidium parvum*), with little focus on waterborne viral outbreaks (Deetz et al. 1984). Some of the viral outbreaks have been attributed to natural water sources, wastewater, and groundwater collected in wells (Borchardt et al. 2003). Contaminated water used in leafy greens production has been shown to be a vector for human illness. In a study conducted by Okafo et al. (2003), it was shown that contamination rates varied with the weather and growing season; dry seasons showed increased levels of contamination due to the need for increased irrigation.

In a recent study conducted by Leifert et al. (2008), the risk of waterborne contamination was found to increase in the order:

- 1.) Potable or rain water
- 2.) Groundwater collected in deep wells
- 3.) Groundwater collected in shallow wells
- 4.) Surface waters
- 5.) Raw or inadequately treated wastewater.

Studies have shown that the quality of groundwater increases with depth. This is believed to occur because of aquifers. Aquifers occur at various depths; those closer to the surface are more likely used for water supply and irrigation to crops, and are more likely to be contaminated by local rainfall (Oosterbaan et al. 1996). Deep well water has also been found to be contaminated even in confined or seemingly impermeable aquifers that are assumed to be protected from pollution (Borchardt et al. 2007). Overpopulation of areas can cause the aquifer to become overused, therefore lowering the water table and increasing the risk of contamination. More porous aquifers promote a greater amount of transport of microorganisms (Sinton 1986).

Studies have been conducted in the attempt to explain the adsorption of viruses to aquifer materials. The most common theory explaining this is the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, which states that a layer of counter-ions develops around the adsorbent and adsorbate (Gerba 1984, Chattopadhyay and Puls 2000). A larger layer of counter-ions around the adsorbent results in less adsorption by preventing van der Waal forces. Since van der Waal forces occur over short distances, a large counter-ion layer will prevent attraction between the adsorbent and the adsorbate. This theory explains viral adsorption when both the virus and the solid are negatively charged, and is believed to be the primary factor in nonspecific interactions (Vega 2006).

Pathogens are also a concern in surface waters. Contamination of these surface waters generally come from animal feces along with favorable environmental conditions for pathogen persistence. It has been shown that intense livestock production directly correlates with the presence of pathogens in nearby aquifers (Johnson et al. 2003). Surface water contamination can also occur as a result of runoff

from farmland. As shown above, adverse weather (especially flooding events) affects the levels of potential contamination to crops. In a study conducted by Tierney et al. (1977), it was shown that polioviruses can persist in soil that has been flooded by contaminated water and can later be transferred onto leafy greens during cultivation. Additional studies have shown that pathogenic viral, bacterial, and parasitic species can survive for extended periods of time in sediments (Lewis et al. 1986, Burton et al. 1987, Amoah et al. 2005).

Different types of irrigation systems have varying effects on the risk of contamination of leafy greens. Spray irrigation systems can transport aerosolized enteric bacteria and viruses onto the surface of plants (Tetltsch and Katzenelon 1978). Although overhead spray irrigation spreads contamination further than other methods, pathogens on the surface of the plants are subject to more environmental stressors, as compared with pathogens in the soil (Brandl 2006, Jiang et al. 2002); however, irrigation studies show conflicting data. For example, in a study conducted by Bastos and Mara (1995), no differences in contamination levels were found between spray and ditch irrigation. In a study conducted by Song et al. (2006), it was shown that contamination levels were higher in a ditch irrigation system than in the spray irrigation; however, the general belief is that subsurface irrigation lowers the risk of pathogenic transfer to leafy greens (Hamilton et al. 2006).

The amount of time between irrigation and harvest also influences the level of pathogens present on the surface of the produce; pathogens decline as time increases between the abeyances of irrigation to harvest. Enteric viruses on the surface of produce decrease almost daily following the cessation of irrigation (Amoah et al. 2005).

Human pathogens can also be transferred to leafy greens via contaminated pesticide solutions. The leafy greens are at a greater risk of contamination if the pesticide is applied close to harvest (Guan et al. 2001). Various bacteria (namely *Pseudomonas*, *Salmonella*, and *E. coli*) can survive in commercial insecticide, herbicide, and fungicide formulations that are used in fresh produce production (Ng et al. 2004). It is also important to keep surfaces that come into contact with fresh produce clear of pathogens, including tools and machines used in harvesting; washing of these instruments should be done using water of good microbiological quality.

The current guidelines in place for the quality of water used to irrigate crops are based on the presence of coliform bacteria and *E. coli* (WHO/FAO 2008). Until better methods of detection of human pathogens are developed, these guidelines will remain in place. Researchers are currently working on developing methodologies that are inexpensive and can be used in the field. In addition to the irrigation water quality being assessed, chemicals applied to crops should also be mixed with potable water. Chemical solutions that are stored prior to or between applications should also be kept at a cool temperature to limit the amount of pathogen growth. When sampling groundwater, surface water, and reclaimed wastewater, it is important to remember that microbial growth and persistence varies between seasons. Therefore, seasonally adjusted testing schemes may be necessary in some regions to ensure a consistent water quality (WHO/FAO 2008). Testing should also occur following a change in the source of water, or in the instance of adverse weather conditions (such as heavy rains and flooding).

1.13 Leafy Greens Post-Harvest

Leafy greens are generally harvested with the roots intact or by cutting the leaves from their roots in the field. After the leafy greens have been harvested, packing and processing can either occur in the field or in designated packinghouses. These steps all increase the risk of contamination of the leafy greens. The risk of contamination comes from the fact that the fresh produce comes into contact with food handlers, harvesting tools, and rinse water. When leafy greens are harvested, the leaves or plants may be hand-sorted, and then may undergo simple processing, such as the removal of outer leaves, the removal of the core, or washing. The harvested produce may be prone to soil contamination; the higher the levels of pathogens present in the soil, the greater chance of contamination. Likewise, if the produce is grown hydroponically, the risk of contamination comes from the water it was grown in. If the produce is then transferred to a machine for processing, there is a risk of bruising and damage, which could open up surfaces for contaminants to internalize (Takeuchi et al. 2000). The leafy greens are then placed into containers for transportation. All of these steps present multiple risks of contamination from food handlers, to contact with surfaces, or water and the environment itself (WHO/FAO 2008). Field coring and trimming is a relatively new process, leading to the need of additional research in the following areas (taken from WHO/FAO 2008):

- 1.)The potential for pathogen internalization through the cut/bruised surfaces during harvesting
- 2.)The effect of field coring and trimming on pathogen contamination and its persistence on the harvested produce

3.)The effect of current post-harvest handling conditions, such as holding time, temperature, atmospheres, and spray water.

Packing poses a problem in that, depending upon the location of the packing, environmental factors such as dust and wildlife can be sources of contamination. Unfortunately, there is little to no physical evidence of microbiological contamination to fresh produce, so laboratory testing must be conducted; however, testing of produce incurs additional costs to packinghouses, which may not be feasible for smaller operations. After the produce is packaged, it is quickly transported to a cooling facility and vacuum cooled to 5°C or lower; however, the time between packaging and cooling varies depending upon the distance of the field to the cooling facility. If we add this fact with coring of the produce, contamination can be readily introduced to the produce and can become internalized; internalized microorganisms are difficult to remove.

If contaminated produce was packed in the field, packinghouses can be at risk for contamination. In a study conducted by Johnston et al. (2006), 11 packinghouses in the United States and Mexico were sampled at various stages of processing for microbial contamination. When testing the product directly, the levels of microorganisms were low. When the receiving bins (those holding the produce prior to washing) were sampled, the microbial levels were significantly higher. In addition, it was found that microbial contamination increased as the produce moved from conveyor belts to the final boxes. This study concluded that contact surfaces were a source of contamination, and cited the importance of proper equipment cleaning. In these packinghouses, it is important to remember that poorly cleaned equipment provide excellent breeding grounds for pathogens (Stafford et al. 2002). In

a study conducted by Prazek et al. (2002), water in packinghouses was tested. It was found that chlorinated, potable water was the best for controlling levels of microbial contamination.

Processing of leafy greens in a factory setting also plays a role in potential contamination. This type of processing may include removal of damaged leaves, coring, cutting, washing, sanitizing, and packaging. The majority of the contamination that is encountered at the processing step is due to residual contaminants from harvest or from food handlers (Nguyen-The and Carlin 1994). When the fresh produce is being processed, it comes into contact with many surfaces. If the produce is already contaminated, the risk of spreading the contamination to other produce is high. When this happens, it is difficult to trace the contamination back to the source. Plant surfaces damaged during processing hold a risk for infection as well; internalization of pathogens is just as likely during processing as it is during harvest (Takeuchi et al. 2000). In a study conducted by Lowry et al. (1989), it was found that a mixture of contaminated food handlers and dirty shredding equipment led to an outbreak of HAV in shredded lettuce.

The washing and sanitizing steps have the potential to reduce the overall microbial load on fresh produce, but it will not remove contamination (WHO/FAO 2008). Studies have estimated that washing produce with sanitized water results in a 1 to 2 log reduction of bacteria (CCFRA 2002). The most common sanitizing agent used is chlorine or chlorine-based products, generally at concentrations of 20 to 100 ppm. Sanitizing is conducted only after debris and organic matter has been removed from the produce to increase the effectiveness of the sanitizer. Research has found that 100 ppm chlorinated sanitizers removes 1 to 2 logs of viruses, which is similar to

the removal of bacteria (Seymour and Appleton 2001). This study also showed that agitation during sanitizing removed slightly more microorganisms, and that two minutes was the threshold of time that sanitizing had maximum benefit; however, if the produce was contaminated with pathogenic virus to begin with, it is highly likely that sanitizing, although removing 1 to 2 log of the virus, would provide little benefit, since there would still be enough virus particles remaining on the produce to cause infection. Where the contaminant is attached also affects the results of the sanitizers; microorganisms are easier to remove from the surface of produce than when internalized. If the pathogen has been internalized, it is suggested to raise the temperature of the sanitizer by 10°C to achieve a positive differential, minimizing the uptake of fresh water through cuts or damages to the produce (Bartz and Showalter 1981, Zhuang et al. 1995).

Packaging also influences contamination risks. If produce is packaged under hygienic conditions directly after sanitizing, the risk for microbial contamination is low. Packaging generally maintains a high humidity for fresh produce to expand the shelf-life of the product; however, if the produce is contaminated prior to packaging, the high humidity in the packaging will allow for proliferation of the contaminants (Brandl and Mandrell 2002). Produce stored in high humidity packaging at room temperature for less than 24 hours can support the growth of pathogenic bacteria such as *E. coli* and *Listeria monocytogenes* (Abdul-Raouf et al. 1993). This study suggested that the shelf-life of the product does not necessarily affect the amount of potential pathogen contamination if kept at room temperature. This same study also found that fresh-cut produce generally has half the shelf-life of the whole counterparts, but more data would be necessary to confirm this. Additional

studies have shown that packaging with 60% relative humidity or less will quickly inactivate pathogens on the surface of produce; however, high humidity packaging can result in condensation at the bottom of the package, which can be a breeding ground for pathogens, especially bacteria (Valentin-Bon et al. 2008). In addition, if the packaging materials do not have good permeability, the CO₂ concentrations within the packaging may increase, selecting for anaerobic pathogens (Carlin et al. 1996).

Regardless of the packaging materials used, other factors, such as initial bacterial numbers and temperature, play equally important roles in the safety of the produce (WHO/FAO 2008). For example, refrigeration will not remove contaminants from the produce, but it may slow contaminant growth (Abdul-Raouf et al. 1993). The data collected from this study suggested that temperature is an important factor controlling pathogenic growth, and maintaining the cold chain is a key factor determining food safety. Cold-chain operations begin directly following harvest. This primary cooling step employs the use of ice in the field, forced-air cooling, hydrocooling, water spray vacuum cooling, or vacuum cooling with no water spray (WHO/FAO 2008). After the produce has been harvested, the cold-chain continues through the washing, cutting, and packaging stages; this can be maintained through cooling rooms in the packinghouses. After the produce has been packaged, transport to merchants is the next step in the cold chain, which is generally maintained by refrigerated transport or the use of ice or dry ice that is placed in between the produce to keep the temperature low. Once the produce reaches the distributor, the cold-chain risks the chance of being broken between the market and the consumer's home.

Although the maintenance of the cold chain minimizes the growth of bacteria, parasites and viruses are generally not influenced by lower temperatures

(Harris et al. 2003). If bacteria are required in large amounts to cause infection and disease, refrigeration will keep them at bay; however, if low levels of infectious doses are required for disease, refrigeration may not be able to hinder the growth enough to prevent this. In addition, if the bacterium is psychotrophic, cold-chain conditions may allow for proliferation and an increased chance of infection (Nguyen-The and Carlin 1994). Viruses survive on fresh produce longer in colder temperatures, with some persisting longer than the actual shelf-life of the product (DeRoeve 1999). In a study by Badawy et al. (1985), it was found that rotavirus can survive on lettuce, radish, and carrots for 25 to 30 days at 4°C.

1.14 Current Foodborne Virus Detection Methodologies

Gastroenteritis infections are generally diagnosed via detection of pathogens in stool samples; this is an easier and much rapid method than measuring the antibody response in serum (Koopmans and Duizer 2004). In the past, stool samples were scanned under an electron microscope for diagnostic purposes; although this method is widely used, it is an insensitive method (Atmar and Estes 2001). Advances in epidemiology and detection methods in the past 20 years due to the increased attention to foodborne viruses have resulted in new methodologies that have been developed for the detection of these viruses in a number of foods and water sources. Because many foodborne viruses are environmentally stable, the development of these methodologies has been important for controlling and preventing outbreaks (Jaykus et al. 1994). Additionally, foodborne viruses are resistant to many methods traditionally used to control pathogenic bacteria, and generally have low infectious doses (Haas 1983, DuPont et al. 1995). These factors allow for virtually any food to pose a threat of transmission of foodborne viruses.

Foodborne viral illnesses are generally under-reported. Most of this information comes from outbreak investigations by state and local health departments is given voluntarily and is not generally comprehensive (Jaykus 1997). Additionally, since the investigations are conducted following the outbreak, many potential samples and sources of information have already been consumed or thrown away prior to the actual investigation, limiting the span and duration of the investigation.

Many of the current viral detection methods used in many countries deal directly with bivalve mollusks and have been developed by accredited research institutions (CDC 2008). Newer methods are being developed for other food products, such as fresh produce. As these methods are being developed, validation is also a concern. Once a methodology is validated, standardized instructions are distributed detailing the proper handling and investigation of a foodborne virus outbreak (Scherer et al. 2009). The current number of detection methods for foodborne viruses in a multitude of different instances shows the increasing recognition of the significance of foodborne viruses as causes of disease (CDC 2008).

Foodborne illnesses caused by enteric viruses can be suspected epidemiologically by studying the incubation period and duration of illness, symptoms of the illness, and the absence of typical bacterial samples (Kaplan et al. 1982). Confirmation of viral particles in the laboratory may be found with antibodies specific to the virus, or the presence of virus particles, nucleic acids, or antigen in a sample; immune electron microscopy, radioimmunoassay, and enzyme immunoassays are also employed (Jaykus 1997). Enzyme-linked immunosorbent assay (ELISA) is generally used for the detection of group A rotaviruses, adenoviruses, astroviruses, and some noroviruses (Koopmans and Duizer 2004). On the other hand, non-group A

rotaviruses, sapoviruses, and other noroviruses are detected using reverse-transcriptase polymerase chain reaction (RT-PCR) assays. Norovirus detection fits into both of these detection methodologies due to the fact that there is variability within the norovirus genome, not allowing it to fit within a single detection test. Hepatitis viruses generally undergo testing with the detection of specific IgM antibodies.

Different methodologies have differing detection limits, ranging from a few particles in RT-PCR, to a million particles per gram in stool samples under electron microscopy. Viruses are typically found at low levels in contaminated foods, and therefore may be difficult to examine; the application of these assays are limited because of low detection limits of 10^4 to 10^5 particles/mL and because some viruses have not successfully been cultured *in vitro* (Hedberg and Osterholm 1993). The viruses that have not been able to be cultured must be detected directly in food extracts, bringing about problems in standardization, inhibition of enzymes used in RT-PCR, and false-positive tests (Lees 2000). In addition, viruses are shed in almost equal amount in both symptomatic and asymptomatic carriers; therefore, these tests can only determine the presence of an infection, and not necessarily the severity (Koopmans and Duizer 2004).

Although molecular-based assays have been developed that can determine the presence of viral nucleotide sequences, large samples of the foods of interest are required to collect enough virus to be detected (CDC 2008). Unfortunately, even the detection of these nucleotide sequences might not be enough; these assays do not count viable versus inactivated viruses, just the amount of genetic material floating around. This factor will alter the results of some assays and cause readings that are higher than the viable viruses actually present. In addition, the use of live cells for

viral growth is necessary. Another concern with these methodologies is the presence of food particles which might interfere with or even inhibit the detection of these foodborne viruses. Newer detection methods based upon immunologic and molecular analysis are currently being developed for widespread use (Jaykus 1997). Another factor that must be taken into account is the infection source. If a food is contaminated by a contagious food handler, the virus will most likely only be seen at the surface of the product; the same can be said for cross-contamination and infected irrigation water. It has also been suggested that some food sources will take in foodborne viruses through the root and can travel to the part of the plant that is ingested, although not much research has been conducted to test this.

Methods are currently being developed that enhance viral extraction and concentration prior to reading; unfortunately, concentration methods are usually long processes with little yield. There are two main methods for the concentration of human enteric viruses from foods: extraction-concentration and adsorption-elution-concentration (Jaykus 1997). The first method is sample preparation, which is usually a combination of filtration, centrifugation, adsorption, elution, solvent extraction, precipitation, and organic flocculation. This method removes as much of the food products as possible and concentrates the virus. Effective viral concentration results in a high recovery of infectious particles in a low-volume solution free of cytotoxic materials. These methods were derived by the removal of viruses from shellfish tissues, following filtration, precipitation, polyelectrolyte flocculation, and solvent extraction (Jaykus et al. 1994). The adsorption-elution-precipitation methods have been favored due to a 10 to 90% virus recovery (Sobsey et al. 1978). The sample manipulations that follow the extraction of the virus depend on the behavior of viruses

to act as proteins in solutions, to co-sediment by centrifugation when adsorbed to larger particles, and to remain infectious at extremes of pH and in the presence of organic solvents (CDC 2008). Sometimes, a method called co-extraction is employed. This method uses a processing control virus that is similar to the target virus in physio-chemical characteristics, is virtually non-pathogenic, and be absent from the sample under environmental conditions.

After the viruses have been concentrated, ideally they would remain viable to infect mammalian cells in culture; unfortunately not all foodborne viruses have been found to replicate under laboratory conditions, or will replicate poorly (Jaykus et al 1994). Therefore, the nucleic acids are generally extracted for analysis. The *in vitro* enzymatic amplification with polymerase chain reaction allows for the amplification of a single nucleic acid sequence and is specific and sensitive. Viral nucleic acid detection can also be determined via RT-PCR, real-time RT-PCR, nucleic acid sequence-based amplification (NASBA), or microarrays. Viral RNA is most often isolated and purified from the sample prior to RT-PCR, although methods have been developed that captures the virus with specific antibody, followed by nucleic acid amplification by RT-PCR (Desenclos et al. 1991). Another approach takes the intact virus particle and concentrates and purifies the sample taken directly from the food matrix, resulting in sample volume reduction and removal of inhibitors, followed by subsequent heat release of viral nucleic acid from the virion capsid and RT-PCR (Chung et al. 1996).

Nucleic acid amplification steps have been replaced with real-time methodologies which are more time efficient to the analytical process and minimize the risk for cross-contamination. Unfortunately, leftover food particles may act as

inhibitory substances, which may alter the analysis. An internal amplification control (usually ssRNA) has been used to counteract this, alerting the researcher of false positives that may be found due to residual food. Analysts worldwide are continually looking for methods of virus detection that are simple, sensitive, and practical, removing the inhibitory effects of residual food particles.

One method that is not currently widely used, but may be in the future is the use of bacteriophages (Lees 2000). Bacteriophages are viruses that infect and replicate in bacteria; these are prevalent in human stool samples, and are similar to viruses that are pathogenic to humans. Similarities in structure, behavior, and stability between bacteriophages and enteric viruses may provide valuable assessments of exposure in stool samples. Although the presence of bacteriophages does not definitively prove the presence of enteric viruses, it would be a stepping stone in the right direction (Slomka and Appleton 1998). Bacteriophages of interest include male-specific coliphages, which have been shown to be a good indicator of the effectiveness of composting due to its resistance to increased temperatures (Mocé-Llivina et al. 2003). Although this bacteriophage is a good indicator for many enteroviruses, it cannot predict the behavior of HAV or noroviruses (Graff et al. 1993); however, this brings about the possibility of using phages as surrogates in evaluating the antiviral effectiveness of current processes (Mariam and Cliver 2000).

Currently, alternative PCR methods are being developed. These methods need to be representative of the sample and sensitive to low levels of contamination while lessening the need for large samples of contaminated food to isolate the viruses. In addition, these approaches need to be efficient in providing concentrated viruses that are free from impurities from the food. Methods are currently being developed

that are inexpensive and easy, which could be available in laboratories around the world. This would allow for opportunities to monitor food safety and prevent outbreaks. Besides improving current virus detection methodologies, it is also important to educate the general public on food safety concerns.

The development of rapid detection methods will also allow for a revamping of critical control points in the Hazard Analysis Critical Control Points (HACCP) approaches to food safety and thereby control the transmission of foodborne illnesses (Jaykus 1997). If we develop more rapid detection quantitative methods, prevalence of contamination and dose-response relationships will be more readily assessed. Rapid quantitative assays will improve risk characterization and reduction, as well as improve overall surveillance of potential foodborne outbreaks. These approaches have the potential for obtaining the information necessary to assess risks, control disease, and improve public health, creating an overall safer farm-to-fork continuum.

1.15 Risk Management and Associated Challenges

Since fresh produce has a relatively short shelf-life and does not encounter much processing prior to consumption, post-harvest risk management is difficult, but important. The most common point of contamination for fresh produce is at the stage of primary production, at harvest (CDC 2008). There are currently many methodologies in place to control for bacterial contaminants; however, these methods do not necessarily detect or control viral contamination. Because of this, growers of fresh produce must monitor the quality of the water used for irrigation, fertilization, and pesticide application during all stages of growth and production. Therefore, this water should be tested regularly for the presence of viral contamination. If the water

is found to be unsuitable, decontamination methods or another water source should be employed. The personal hygiene of the food handlers must also be monitored to reduce the risk of foodborne virus spread.

Most foodborne viruses found on fresh produce will outlast the shelf-life of the product. This must be taken into account when proposing methods for the reduction or inactivation of viral contaminants. Unfortunately, there are currently large data gaps about the specific agricultural practices used in many countries and the extent to which countries exporting fresh produce adhere to GAP programs (CDC 2008).

Risks of foodborne viral contamination decreases with the amount of knowledge and information available pertaining to effective hand and environmental decontamination procedures; this varies among countries (CDC 2008). An important part of risk management is to have hand-washing stations available to food handlers at all times, and for these food handlers to maintain an overall high degree of personal hygiene. When a food-handler is found to be shedding virus, it becomes difficult to assess the degree of contamination based on the amount of virus being shed and the amount of exposure to the food preparation areas. This causes problems for risk management, in addition to the lack of understanding for shedding patterns of an infected food-handler. Currently, testing as a means of control is not available because of the limited availability of diagnostic methods outside of laboratory conditions, as well as a lack of culture systems for most wild-type enteric viruses (CDC 2008).

Overall, control of viral contamination of fresh produce begins with prevention, both in water treatment and food-handler hygiene. This process is more

effective than attempting to destroy the pathogen following contamination. In addition, most current practices focus on bacterial pathogens, which do not necessarily work on viruses. Monitoring activities at pre-harvest are currently being developed that could provide useful information for the management of potential viral contamination (CDC 2008). This process could develop a new indicator to test for the presence of human pathogenic viruses on fresh produce. Although an appropriate indicator has not yet been found, the future is promising, as technologies have been developed that detect viruses in shellfish and water. In addition, recent foodborne outbreaks have kicked educational programs into gear to prevent these outbreaks from recurring. The increasing amount of information being distributed is of great importance in addressing the problem of foodborne viruses.

Although there are not currently any internationally recognized standards for irrigation water used on fresh produce, development of such protocols would potentially increase the overall reduction of foodborne viral contamination pre- and post-harvest (WHO 2006). These protocols would require growers of fresh produce to adhere to international requirements for harvesting and exportation maintaining an overall higher quality of products with less risk of contamination involved.

Food handlers also play an important role, and should be scrutinized under the same sort of standard regulations. These regulations would include the removal of infected employees and guidelines for their return to work (CDC 2008). Also included in these regulations would be standards for personal hygiene, including proper hand washing techniques. Development of new hand and produce sanitizers could also be beneficial, as well as educational programs to inform employers and

employees on current risks from foodborne viruses. Risk management is of utmost importance in the reduction of foodborne viral outbreaks.

1.16 Survival of Enteric Viruses on Fruits and Vegetables

Enteric viruses are able to survive on leafy greens for extended periods of time. In a study conducted by Dawson et al. (2005), the MS2 bacteriophage was found to survive on tomato, cabbage, carrot, lettuce, parsley, pepper, and strawberries at 4°C and 8°C for more than 87 days with a reduction of less than 2 logs throughout the sampling time. In another study, poliovirus was found to survive on lettuce and radishes for more than three weeks (Tierney et al. 1977). Kurdziel et al. (2001) also studied the persistence of poliovirus on romaine lettuce, green onions, white cabbage, fresh raspberries, and frozen strawberries at 4°C (-20°C for the frozen strawberries) for two weeks. Poliovirus was found to decrease by 90% on lettuce after 11.6 days, 14.2 days on white cabbage, 8.4 days on frozen strawberries, and did not decline on fresh raspberries or green onions. Croci et al. (2002) found that HAV survives on lettuce, fennel, and carrot at 4°C (discussed below). Studies on enteric virus persistence by Allwood et al. (2004) found persistence of MS2 and feline calicivirus (FCV) on lettuce and cabbage at 4°C, 25°C, and 37°C. At 4°C and 25°C, MS2 survived 12 days, while FCV persisted for 5 days, while at 27°C, MS2 survived for 12 days and FCV survived for 3 days.

A study by Stine et al. (2005) examined the effect of humidity on viral and bacterial agents in a controlled environment chamber. It was found that bacteriophage PRD1, HAV, and FCV survived on cantaloupe better in dry than in humid conditions. On lettuce, bacteriophage PRD1 and HAV survived longer in dry conditions than in humid conditions, while FCV survived better in humid conditions. On bell peppers,

HAV and FCV survived longer in dry conditions, and bacteriophage PRD1 survived longer in humid conditions. This study showed that enteric viruses are able to survive on fresh produce in both humid and dry conditions, and that both the physiochemical stability of the viral capsid as well as the epiphytic community to utilize viruses as carbon or nitrogen sources (Vega 2006). Bidawid et al. (2001) suggested some importance of epiphytic communities on virus survival with modified atmosphere packaging (MAP). This study found that although MAP extends the shelf-life of fresh produce, HAV survived better on MAP lettuce than on unpackaged lettuce. It is believed that these data were obtained due to microbial predation; the use of carbon sources by epiphytes on leaves may be important for virus persistence (Bidawid et al. 2001, Vega 2006).

Studies have supported this theory; although simple sugars are readily available on leaves, carbon sources seem to support epiphytic growth. Mercier et al. (2000) found that the initial concentration of sugars allows for a rapid proliferation of *Pseudomonas fluorescens* on the leaf which quickly plateaued with sugars remaining on the leaf surface. This study suggested that instead of the sugars present, epiphytes utilize carbon sources on plant surfaces. In addition, *Pseudomonas* spp. grows rapidly and utilizes carbon and nitrogen sources available on plant surfaces. Many species of *Pseudomonas* isolated from plants have been found to utilize amino acids and possess hydrolases that can make use of sucrose, maltose, trehalose, and xylans (Paulsen et al. 2005). Current food preservation techniques focus on inactivation or reduction of the microbial load to increase shelf life; this has resulted in the selection of microbes that can survive under adverse conditions and are able to utilize any available source of carbon and nitrogen (Vega 2006).

The current most widely used treatment for washing fresh produce is between 50- to 200-ppm chlorine, which effectively decreases bacteria and parasites. Other treatments include bleach, hydrogen peroxide, Tsunami 100, sodium bicarbonate, and electrochemical inactivation (Beuchat and Ryu 1997, Drees et al. 2003). Viruses do not have the lipid membrane of bacteria, and are therefore more resistant to disinfectants; however, in a study by Malik and Goyal (2006), sodium bicarbonate was found to reduce FCV on some environmental surfaces; although FCV is used as a norovirus surrogate, the authors note that it is not known how closely FCV mimics norovirus infections in humans, as FVC generally infects cats. Another study conducted by Gulati et al. (2001) tested a number of disinfectants for their effectiveness against caliciviruses and found that all eleven tested was ineffective in inactivating caliciviruses.

Overall, sanitizers are developed based on bacterial standards. Viruses do not interact with their environment and remain in stasis until activated by a host cell (Vega 2006). Viral survival is due to structural and genetic stability; these characteristics make enteric viruses difficult to remove from the surface of fresh produce. According to the CDC, the most effective method of removing/inactivating viruses from surfaces is a solution of approximately 1000 ppm of chlorine, which is between 5 and 20 times the FDA recommended treatment for fresh produce (CDC 2002). Because of this, there is currently no effective treatment of washing fresh produce to remove enteric viruses without reducing quality (Vega 2006).

1.17 Fertilizers

Farmers in the United States have a number of options when providing nutrients to agricultural crops. Many operations use readily available, organically rich

manures, while others use commercially produced mineral fertilizers (Spicer 2002). Biosolids are also a feasible option, although not widely used. Each fertilizer has its own set of benefits and detriments, including agricultural properties, metal accumulation in soils, plant uptake of metals, contamination of groundwater by organic compounds, and potential environmental effects from pathogens.

Every year, approximately 120 million dry tons of animal manure, 50 million dry tons of mineral fertilizer, and 2.8 million dry tons of biosolids are applied to agricultural crops (Spicer 2002). Due to its wide availability, animal manure is 40 times more common to be used as a fertilizer worldwide. Studies have shown that the risks associated with biosolids are less than or equal to those associated with animal manure (Moss et al. 2002). Both manures and biosolids contain similar amounts of important macronutrients, such as nitrogen (1-10.8% of dry weight), phosphorous (0.7-7.5% of dry weight), and potassium. Chemical fertilizers, on the other hand, contain 15-82% nitrogen and 8-76% phosphate; the remaining nutrients are added to be specific to plant needs. Because of the high levels of macronutrients available in chemical fertilizers, less material is required.

Manures and biosolids deliver organic material to the soil, improving the quality for future crops. The addition of organic material has been found to improve the soil's water retention, water infiltration, bulk density, and porosity (Moss et al. 2002). Studies have found that following the application of manure or biosolids as much as a 26% reduction in irrigation needs along with increased yields have been found (Spicer 2002). Organic materials are also negatively charged, which also help to improve the pH of the soil, increasing the cation exchange capacity. This allows the soil to retain positively charged nutrient ions such as ammonium and calcium.

Additional studies conducted by Moss et al. (2002) have shown that organic nitrogen added to soil will undergo mineralization which will provide plant-available nitrogen in the soil.

Micronutrients are also present in biosolids and manures. Studies have found that biosolids generally contain higher concentrations of these micronutrients than cattle manures, but have similar levels as are found in swine and poultry manures (Spicer 2002). Chemical fertilizers, on the other hand, contain elevated levels of metals (Table 2), especially cadmium, which is used in phosphate fertilizer production.

The US Environmental Protection Agency (EPA) created the 40 CFR Part 503 Rule, categorizing biosolids into Class A and Class B. These different classes were based on the level of pathogenic organisms in the material. Class A biosolids have undetectable levels of indicator pathogens for *Salmonella*, enteric viruses, and helminth ova; this is achieved by heating, composting, digestion, or increased pH (Spicer 2002). These treatments change the composition of the biosolids, creating a pellet or granular substance, which can then be sold for personal use for lawns and flowerbeds (Foess and Fredericks 1995). Class B biosolids are less strictly regulated, and must contain less than 2 million colony-forming units or fecal coliform per gram of total solids (Spicer 2002). Pathogen levels in Class B biosolids have been reduced to levels that protect public health and the environment. These biosolids are used for crop harvesting and grazing animals, and undergo treatment in a wastewater treatment facility as well as heating, composting, digestion, or increased pH. Studies have not found any environmental effects from biosolids due to the reduced number of

pathogens; however, groundwater and surface water contamination has been found as a result of manure (Moss et al. 2002).

Animal manures are not typically treated to reduce the amount of pathogens, and are used in greater quantities than biosolids. Levels of indicator pathogens in manures are higher than those found in biosolids; studies have found between 5 and 30 million fecal coliform colonies per gram (Spicer 2002). Moss et al. (2002) also found that healthy cattle manure may contain as many as 10 million *Salmonella* spp. per gram of feces.

Most studies on fertilizers have been on the economic benefits and have focused on the savings possible from replacing chemical fertilizers with manures or biosolids (Spicer 2002). Further research is necessary to educate the public on manures, biosolids, and chemical fertilizers. Some topics for consideration of further research include manure content and nutrient availability, potential pathogens in chemical fertilizers and detection methodologies, long-term field studies of all materials under laboratory conditions, and ecological assessments of soil amendments and chemical fertilizers, as well as potential health effects (Moss et al. 2002).

1.18 Hepatitis A Virus (HAV)

Hepatitis A was recognized as a distinct form of hepatitis during World War II, but studies conducted did not prove that HAV existed until many years later. What made this discovery difficult is the fact that prevalence of HAV varies from country to country, usually correlating with the standard of living. In the mid-1990's, there was a noticeable shift from HAV prevalence from childhood to adulthood; improved living conditions reduced the number of infected children per year, which resulted in a higher susceptibility of adults to the virus (Melnick 1995). Prior to this,

HAV was most often found in intravenous drug users, institutionalized persons and their caretakers (because of close living conditions), and among travelers whom traveled from areas of low HAV prevalence to areas of high HAV prevalence. Symptoms of HAV, mostly jaundice, were recorded as early as the 5th century B.C.; although a number of pathogens can cause liver disease that leads to jaundice, HAV is the most common culprit. Major outbreaks of HAV were recorded in Europe in the 17th and 18th centuries, and in the 19th century, the cause of jaundice was termed “acute catarrhal jaundice.” It was around this same time that epidemics of jaundice were frequent, especially in army regiments; because of this, jaundice was given the nickname “campaign jaundice.” In 1923, Blumer suggested that the catarrhal jaundice was actually a result of infectious hepatitis. During World War II, a serum hepatitis was discovered, and its agent was labeled hepatitis B virus (HBV); this was known to be distinct from the epidemic-causing virus, which was called hepatitis A (Melnick 1995).

Following the distinction between HAV and HBV, studies were conducted using human volunteers (Havens and Paul 1959). Studies conducted by Krugman et al. (1959) resulted in the development of the HBV vaccine. This study also showed that there was little heterologous immunity between HAV and HBV, suggesting that any newly developed vaccine must be specific for either HAV or HBV. Additional studies were conducted with human volunteers inoculated with virus-containing serum; these studies showed that HAV infections in children were much milder than the same infection in adults (Melnick 1959). These human trials were highly valuable in determining the incubation period of HAV, which was found to range from 15 – 40

days, with an average of 25 days. In addition, HAV was proven to be an enteric virus that can remain infectious following heating at 56°C for half an hour.

In the early 1960s, studies were conducted determining if transmission was possible between humans and non-human primates, or vice versa. Following many failed attempts at transmitting HAV to a number of other species, Deinhardt et al. (1967) successfully transferred HAV to a marmoset, a non-human primate. When a report of a new strain of virus similar to HAV, called the GB agent, was found in a surgeon, it was believed that the virus had been transmitted during surgery; however, the GB agent was found to be practically identical to HAV. This puzzled researchers. In 1969, Parks and Melnick attempted to delineate the differences between HAV and the GB agent. It was found that marmosets actually carry latent GB agent, which can cause hepatitis in humans. Not satisfied with these results, Boggs et al. (1970) inoculated marmosets with a strain of HAV that was previously injected into human volunteers. This study proved marmosets to be suitable for transmission studies of HAV, and that the GB agent was distinct from HAV, not being recognized as any of the five agents that cause human hepatitis (discussed in Purcell 1993). Since this study, more than 35 outbreaks of hepatitis A have been attributed to transfer from primates, usually chimpanzees, to humans that work closely with them (Melnick 1995). Unlike marmosets, it is believed that these chimpanzees contract HAV while in captivity, and are then able to transfer the virus to their caretakers.

In 1973, HAV from infected volunteers from studies with Boggs et al. was visualized with electron microscopy (Feinstone et al. 1973). This visualization allowed for the classification of HAV to be placed in the Picornaviridae family, being distinct from the other four families that the remaining hepatitis viruses belong.

Shortly after visualization, HAV was cultured in primate cell cultures, with little damage reported in the cell following several serial passages (Funkhouser et al. 1994). Growing HAV in cultures of cells increased the chances of diagnostic testing to be made available to hospitals and a wider range of laboratories. It was also found that serial passage of HAV causes it to lose virulence for the liver, which led to the development of HAV vaccines using formally inactivated HAV. In 1995, the first HAV vaccine was produced by GlaxoSmithKline, and another vaccine became available the following year by Merck, both of which were FDA approved and equally effective (IAC 2009).

Trends can be seen in hepatitis A outbreaks. Peak season for hepatitis A seems to occur in autumn, generally with higher prevalence in children. This trend is believed to be a result of traveling during the summer. In Europe, it is common to travel to the Mediterranean during the summer where hepatitis A is more prevalent (Frosner 1990). Trends also show that hepatitis A symptoms generally occur within 8 weeks of visiting a higher prevalence country. Other, less common means of transference of HAV are blood transfusions and contaminated needles/syringes; however, since there is not a persistent HAV carriage state, the latter is highly unlikely.

Researchers have found that the epidemiology of hepatitis A is best determined via humoral antibodies. In developing countries, antibodies will appear early in life following exposure. These antibodies are generally detected throughout adulthood, although this is not only the case; it is believed that developing and maintaining anti-HAV antibodies requires more than one exposure to the virus (Melnick 1995). This is seen in countries with poor sanitation, where antibodies are

present in children, and increase throughout adulthood, suggesting multiple instances of exposure. On the other hand, developed countries show little exposure of children to HAV, leading to only moderate prevalence of the anti-HAV antibodies as adults. Studies have shown that between 1965 and 1975, the prevalence of these antibodies decreased from 51% to 11% in ages 20-29, and decreased from 88% to 46% in those ages 30-39 (Frosner 1990). These same trends began in the Scandinavian area as early as the 1920s. In more isolated regions, the presence of anti-HAV antibodies varies greatly, based on epidemics of hepatitis A. When an epidemic of hepatitis A reaches these isolated areas, virtually everyone in the community becomes infected, which will result in the amount of people left to be infected close to zero, and a virtual disappearance of HAV infections. When a new generation is born in these areas, the anti-HAV antibody presence decreases because of a lack of exposure, resulting in an increasing population of HAV susceptible people; the next hepatitis A outbreak will start the cycle over again (Frosner 1990).

The trends in the presence of anti-HAV antibodies between children and adults follow a trend similar to that of poliomyelitis, also from the picornavirus family. In a study conducted by Paul et al. (1952), the antibody patterns poliomyelitis was measured in northern Alaska (isolated region), Cairo (substandard level of sanitation), and Miami (high level of sanitation). In Cairo, it was found that antibodies to poliovirus developed earlier in life than in Miami, and those studied in northern Alaska developed these antibodies much later in life than those in Miami. Although standards of living in these remote areas in Alaska are also substandard, antibodies were only found to develop following an epidemic. As with the anti-HAV antibodies

of those in isolated regions described above, the trend of poliovirus in the northern Alaska portion of this study followed a similar ebb and flow.

Foods have been implicated in the transmission of HAV over the course of multiple studies, with molluscs and fresh produce being the main carriers (Halliday et al 1991, Lees 2000, Beauchat 2006). Recently, fresh produce has been involved in an increasing number of cases of HAV outbreaks in numerous countries (Dentinger et al. 2001). The incidence of HAV infection varies considerably among and within countries; the risk of HAV infection is proportional with decreased standards of living. HAV is most common in developing countries, becoming endemic (Mast and Alter 1993). In these countries, people are generally infected with HAV in childhood, with symptoms being asymptomatic. This results in the majority of developing countries' adults to develop immunity to HAV. This virus is generally transmitted from person-to-person contact, and outbreaks are rare because of the asymptomatic conditions. On the other hand, in developed countries, HAV is much less common. Since most people in developed countries are not exposed to and infected with HAV in early childhood, many adults are therefore susceptible to HAV infection later in life. Tourism also increases the incidence of HAV infections, especially people traveling from countries with a high standard of living to countries with lower standards of living (Steffen 1992). Since HAV shedding occurs 10 to 14 days before symptoms appear, spreading the virus becomes quite easy, and outbreaks can run rampant. In addition, the incubation period for HAV can be long, which can affect possible associations with foods consumed weeks prior to the onset of symptoms, resulting in difficulties pinpointing the cause of the infection. If these adults are exposed to an HAV infection, the result is commonly much worse; this increases the potential risk of

HAV outbreaks in these regions (Koopmans and Duizer 2004, Pintó and Sáiz 2007). In addition, many HAV outbreaks occur as a result of person-to-person contact. Through vigilance of proper sanitation, as well as increasing HAV vaccinations, hepatitis A may one day become an infection of the past.

1.18.1 Outbreaks

Since the degree of worldwide sanitation is improving, a proportional amount of people are becoming susceptible to HAV infections (Melnick 1995). With an increasing number of susceptible people, the risk of an outbreak due to HAV-contaminated water or food is also increasing; however, roughly 3% of the reported cases of hepatitis A are associated with food or water (CDC 1989).

Viral hepatitis is endemic in Shanghai, China, and because of this, the government set up the Shanghai Hygiene and Anti-Epidemic Center in 1956, which is required to submit notification and registration of all cases of viral hepatitis (Halliday et al. 1991). This system has been effective in reporting cases of viral hepatitis, along with the agent responsible; 45% of cases of viral hepatitis have been caused by HAV. Since the 1970s, trends in incidences of hepatitis A outbreaks have been found; the majority of hepatitis A outbreaks in Shanghai occur between February and March; however, in 1988, this trend was not the same; in fact, hepatitis A outbreaks began a month ahead of schedule, and the number of cases much higher than usual, sickening 4,083/100,000 people, and resulting in 292,301 cases (Halliday et al. 1991, Xu and Hu 1992). The epidemic was over in March. It was believed that this epidemic was the result of undercooked clams. This was only the third clam-related HAV epidemic in China with the first in 1978 in the Zhe-Jiang Province, and the second in Shanghai in

1982 (Yao et al. 1979, Kang et al. 1983). To date, this outbreak has been the largest reported epidemic of shellfish-associated hepatitis A in the world.

Water samples were taken from water supplies in the epidemic areas, and analyzed on the national hygienic index. The attack rates for hepatitis A along with the quality of water samples were compared with water samples taken outside of the epidemic area to determine if there was a causative link between the water supply and the presence of hepatitis A (Halliday et al. 1991). In addition, an in-depth study conducted by the Shanghai Hygiene and Anti-Epidemic Center investigated clam suppliers, obtaining information on the catching, storing, supplying, selling, and purchasing of clams for Shanghai. Samples were taken from the area that was believed to be responsible for the HAV contamination. ELISA was used for all tests for IgM anti-HAV, HBsAg, and IgM anti-HBc. Positive identification of HAV from clam samples were detected via direct immune electron microscopy, nucleic acid hybridization tests, and tissue culture, while stool samples were examined by immune electron microscopy and tissue culture (Halliday et al. 1991, Hu et al. 1989).

The thorough investigations undertaken in this study showed without a doubt that raw or undercooked clams are a prime source of HAV contamination. Because hepatitis A is endemic in Shanghai, doctors are rich in experience in early diagnosis and citizens are well-informed on the disease, both of which resulted in few deaths associated with this outbreak. Since the outbreak in 1988, strict regulations have been employed pertaining to clam harvesting and distribution (Halliday et al. 1991). Because shellfish filter large amounts of water, it is possible that viral levels within the shellfish can accumulate to levels fifteen times greater than what is present in the water (Hu et al. 1986). Because of this, constant testing of waters used to

harvest clams, as well as the monitoring of drainage into these areas has been put into effect. Following the hepatitis A epidemic in 1988, the government forbade the sale of clams in Shanghai, and regulations have been put into effect allowing the Ministry of Agriculture to determine the acceptable areas for the harvest of clams.

Another seafood-related outbreak of hepatitis A recorded in 1988 occurred in August, half a world away. Cases of hepatitis A were reported in Panama City, Florida seafood restaurants and oyster bars. Panama City is surrounded by three bays in which shellfish, especially oysters, grow naturally (Desenclos et al. 1991). The Florida Department of Natural Resources manages the bays, marking them approved, conditionally approved, or prohibited for harvesting shellfish, based on overall water quality. Seven years prior to summer 1988, the bays surrounding Panama City were prohibited for oyster harvesting; in the summer of 1988, oyster harvesting was permitted in one of the three bays. When patients began arriving at local hospitals with symptoms of jaundice, presence of IgM, and an overall diagnosis of hepatitis A, the Florida Department of Health began an investigation. Confirmed cases of hepatitis A in this outbreak were IgM antibody positive to HAV, and consumed seafood 10 – 50 days prior to the development of symptoms.

The rates of contamination were estimated by examining local oyster bar and seafood restaurant invoices where the confirmed cases had eaten. The origin of the oysters was traced back using the bag tag numbers recorded by the restaurants. In addition, the Florida Department of Health received reports from the Florida Marine Patrol on coliform counts in approved harvesting areas, as well as reports on illegal oyster harvesting activities in and around Panama City, in which samples were provided. This study also inspected both approved and prohibited harvesting areas to

attempt to determine if there was a causative agent associated with the water and hepatitis A. Unfortunately, the environmental investigation was not successful in identifying the source of the contamination.

Coliform counts were taken from the water and oyster meat samples. Detection of HAV antigen and nucleic acid was performed by removing the oyster meat and homogenizing the samples and clarified by centrifugation. The supernatant was then tested for HAV antigen. HAV RNA was detected by *in vitro* nucleic acid amplification via PCR. Samples from both legal and illegal harvesting were found to have traces of HAV.

Sixty-one cases of hepatitis A were confirmed during this outbreak, with no deaths; this was the largest oyster-associated hepatitis A outbreak in the United States since the Louisiana outbreak in 1973 that affected 263 individuals (Portnoy et al. 1975). The illnesses lasted from 7 to 49 days, with an average of 21 days, and had an incubation period of 16 to 48 days, with an average of 29 days. The outbreak affected patrons of 11 seafood restaurants and oyster bars in Panama City, as well as one restaurant in Alabama that served oysters harvested in Panama City. The risk of HAV infection was higher when the oysters were consumed raw and when larger numbers of raw oysters were consumed. This also suggests that with an increasing number of raw oysters consumed, there was an increasing chance of eating an infectious oyster rather than increasing the amount of virus ingested (Desenclos et al. 1991); however, there was one case where baked oysters were consumed, and another with raw scallops harvested in the same area as the oysters associated with the outbreak. No correlation was found between the number of raw oysters consumed and the incubation period, the duration of illness, or the rate of hospitalization. Since the

samples obtained from both legal and illegal harvesting sites were positive for HAV, it is possible that the hepatitis A outbreak was more widespread and was just under reported as is the general case.

In another hepatitis A outbreak, frozen strawberries were the source of an outbreak. Between April and June, 1990, 19 students and 2 teachers at an elementary school in Georgia were diagnosed with hepatitis A, after no additional cases had been reported in the two years prior to the outbreak. In addition, between August and September of the same year, an institution for the developmentally challenged in Montana was also plagued by hepatitis A, with 36 confirmed cases after no reports had been filed for the previous 19 months. Those exposed to HAV were interviewed by Niu et al. (1992) regarding symptoms, common exposures, and other risk factors for hepatitis A during the “exposure period” of 2 to 6 weeks prior to the appearance of hepatitis A symptoms. Serum samples were taken and tested for the presence of IgM anti-HAV, finding some asymptomatic cases. Stool samples were also collected and tested for the presence of HAV antigen. HAV nucleic acids taken from the stool samples were analyzed by RT-PCR; the sequences of the Georgia and Montana HAV strains were compared. In addition to those that ate the potentially contaminated frozen strawberries, the food handlers in both institutions were tested for the presence of anti-HAV antibodies; all food handlers in both institutions were clean.

Using information found on the empty packaging, the HAV source was traced back to a processing plant in California, where the strawberries were picked and frozen in 1988, and distributed in the late fall of 1989. HAV RNA sequenced from both outbreaks were found to be identical to one another, yet distinct from other known HAV strains at the time. When reviewing the medical histories of the plant

workers associated with the shift implicated with these outbreaks showed that no plant workers had been reported as having hepatitis A; because of this, it is believed that the contamination came from an infected strawberry picker (Niu et al. 1992).

In February and March 1997 in Michigan, there was a large, foodborne outbreak of HAV. This outbreak caused 213 cases of hepatitis A and an additional 29 cases were reported in Maine, all from the same batch of frozen strawberries. All of the PCR products in the Michigan cases were identical, and 8 of the 10 sequenced in Maine were identical to the Michigan products. In addition, seemingly sporadic cases in Wisconsin (5 cases), Arizona (7 cases), and Louisiana (2 cases) were found to have the same RNA sequences as those in the main outbreak.

Frozen strawberries were suspected as causing the outbreak. FDA officials visited the three growing fields where the strawberries had been picked and found that the water used for irrigation was piped from a river and filtered through sand tanks, and the only available hand washing stations were on trucks that circulated through the fields (Hutin et al. 1999). The pickers were not wearing gloves, and no medical records could be found on the pickers. It is suspected that one of the pickers was shedding HAV, leading to the outbreak.

In June of 1999, the Health Secretary of Rio de Janeiro received reports of 19 cases of hepatitis A occurring in children attending the same public school in Grajaú, north of Rio de Janeiro (Villar et al. 2002). At the end of July, the number of cases rose to 25, six of which were asymptomatic. Since this outbreak occurred at a public school, the Health Secretary required each of the 509 students to be tested for total and IgM antibodies anti-HAV, and water samples were taken from school facilities. Families were also asked to fill out questionnaires concerning the sanitary

conditions of housing, swimming habits, class attendance, and recent exposure to someone infected with hepatitis A.

Of the 509 students tested in this study, 54% of the serum samples were positive for total anti-HAV; an upward trend was found with increasing age. Based on these results and those of the questionnaires, it was found that HAV exposure correlated with family income; those families with higher income had a lower prevalence of total anti-HAV. It was also found that larger families had higher levels of anti-HAV as well. The first case was found to be asymptomatic, which caused the spread of HAV through the school. The next three cases were family members of the first case, all of which continued to go to school. It is believed that person-to-person contact was the most likely culprit for HAV transmission. Although an HAV source was not pinpointed, researchers believed that dirty bathroom facilities and improper hand washing enhanced the spread of the virus.

1.18.2 Outbreaks Associated with Fresh Produce

In 1988, 26,600 cases of hepatitis A were reported in the United States, an increase from 1983 of 9.2 to 10.9 per 100,000 people (Rosenblum et al. 1990). Of these cases, 1,000 were suspected to be the result of contaminated food or water. Between February and March of 1988, a widespread outbreak of hepatitis A occurred in Jefferson County, Kentucky and the surrounding areas. This outbreak affected 202 people, two of which died. All of the cases either tested positive for IgM anti-HAV or were diagnosed with hepatitis A by a professional. Four of the cases in this outbreak were employees of the restaurants infected during the exposure period, and were not a cause of the outbreak. Prior to this outbreak, 12 cases of hepatitis A had been recorded in the previous year. Interviews of the cases found salad or other meals

containing lettuce were consumed at one of three restaurants, which were potentially associated with the hepatitis A outbreak. Two of these restaurants had received fresh produce from the same local distributor.

The FDA and the Louisville Water Company found that people who dined at the first restaurant were 11.6 times more likely to become infected by HAV after consuming green salad, and the risk of acquiring hepatitis A increased with the increase of green salad consumption (Rosenblum et al. 1990). Patrons that did not order salad, but consumed sandwiches with lettuce or tomato were also at an increased risk for HAV infection. In the second restaurant, those who ordered the salad bar were at a 4.4-fold risk for contracting hepatitis A than those that did not consume salad. In both restaurants, no single item could be identified as the transmission factor for HAV; however, researchers were able to determine that either lettuce or tomatoes were most likely the culprit. That the two restaurants in this study only served iceberg lettuce, which is more difficult to thoroughly clean than tomatoes.

When the FDA inspected the distributor facilities, it was found that no cases of hepatitis A were reported among the workers, and the handling of the produce was minimal; in fact, the produce was rarely washed or processed prior to distribution (Rosenblum et al. 1990); however, the iceberg lettuce was grown on farms in Mexico, which were not inspected in this study. It is believed that the lettuce was contaminated prior to distribution. This was the first hepatitis A outbreak recorded in the United States associated with fresh produce that was likely contaminated prior to distribution.

In November and December of 1998, 26 cases of hepatitis A were reported in Ohio with only one case reported in the previous four months (Dentinger et

al. 2001). Sixteen of these cases ate at the same restaurant. Cases were identified by the presence of IgM anti-HAV. Samples were also taken from employees of the restaurant in question, and the employees were questioned regarding the ingredients of each menu item, and methods of food preparation and storage. The samples that tested positive for HAV infection were run through RT-PCR that amplified the entire VP1 region of the HAV genome, and direct sequencing of the PCR products were done. It was found that the RNA sequences of 15 other sporadic cases elsewhere in the United States and one case in Mexico also had identical sequences. There were eight additional sporadic cases of hepatitis A reported in Ohio that were not associated with the outbreak, but the RNA sequences differed from those in the outbreak.

At the end of the study, 43 cases of hepatitis A were reported with 33% being hospitalized (Dentinger et al. 2001). All of the people ate at the restaurant in question, and 95% reported consuming a dish with green onions. Blood samples from all employees working during the exposure were taken, and all were found to be negative for IgM anti-HAV. Since the PCR sequences were identical among cases, it is highly probable that the HAV originated from a single source. It is believed that green onions were most likely contaminated with HAV prior to distribution to the restaurant. If the outbreak was a result of an infected food-handler, it is highly likely that more than just the green onions would have been involved in the outbreak.

Green onions have also been associated with outbreaks of *Shigella* and *Cryptosporidium*, leading scientists to believe that green onions are prone to contamination by enteric pathogens during planting, irrigating, harvesting, and shipping by contaminated workers or non-potable water. Green onions require a high level of handling, which also increases the risk for contamination. The FDA traced

the green onions in this study back to farms in Mexico and southern California; however, it is believed that the contaminated green onions originated in Mexico; the PCR sequences of the outbreak strain were similar to those found in Mexico, where hepatitis A is endemic.

In November 2003, the Pennsylvania Department of Health was alerted of 111 cases of hepatitis A in Beaver County, PA; only one case of hepatitis A had been reported the previous year (Wheeler et al. 2005). Six of the reported cases had eaten at the same restaurant. To be considered for this investigation, patients had to eat at this restaurant within 2 – 6 weeks prior to the onset of illness, and to be serologically confirmed to have IgM antibodies to HAV. The restaurant was inspected, but no safety violations were found, but food workers were reported as ill. In addition, information was collected pertaining to food purchases, storage, and preparation practices during this period. By the end of this outbreak, a total of 527 cases of hepatitis A from the restaurant were recorded, with 13 cases being employees of the restaurant, and three resulted in death. Twenty-six percent of the cases were hospitalized.

Of these cases, 91% had consumed mild salsa during the exposure period (Wheeler et al. 2005). In addition, after examining 102 ingredients, it was found that diced white and green onions were closely associated with illnesses. Green onions were found in other dishes potentially associated with hepatitis A infection, suggesting that green onions may have been the carrier of HAV. The nucleic acid sequences of the cases in this outbreak were identical, as well as those involved in the 3 deaths (Wheeler et al. 2005). The sequences were also 96% similar to HAV strains found in

tourists in Mexico during this same exposure period. In addition, outbreaks involving green onions in 1999, 2000, and 2003 had similar sequences to this strain of HAV.

The FDA traced the green onions to farms in Mexico, which were contaminated before or during packing on farms (Wheeler et al. 2005). It is believed that the outbreaks of 1999, 2000, and 2003 were also the result of green onions grown in Mexico. In November 2003, the FDA issued an import ban on green onions from northern Mexico. Other outbreaks due to enteric pathogens associated with green onions have been reported. Because green onions are handled during harvesting and preparation for packing, contamination can occur at many points; critical points of contamination may be contact with infected harvesters or contact with contaminated water. Green onions from the same farms were distributed to other restaurants across the United States; however, no additional outbreaks of hepatitis A were reported. It is believed that the contamination was confined to a small portion of the harvest (Wheeler et al. 2005).

1.18.3 Studies

In a study conducted by Croci et al. (2002), the adsorption capacity of the surface of various fresh vegetables and the presence of HAV were examined. Samples of laboratory-contaminated lettuce, fennel, and carrots were stored at 4°C and examined at specific intervals via RT-PCR for the presence of HAV prior to and following washing. The samples testing positive were then quantified in cell cultures, and the infectivity was analyzed. The study showed that the three vegetables differed in their adsorption capacity with washing providing a one-log decrease in the amount of virus on the vegetable. Lettuce retained the most virus, most likely because of its larger surface area and wrinkled texture providing greater protection to the HAV.

Fennel and carrot, on the other hand, had less adsorption of the HAV, and more rapid decrease of the virus and quicker inactivation. It was suggested that the low virus count on the carrot sample was due to the presence of a specific substance that have been shown in other studies to exert antiviral activity (Babic et al. 1994). Although washing decreased the amount of virus on the vegetables, it was not completely removed, and would still remain a threat to public health. It should be noted, however, that the RT-PCR used in this study was only able to detect the nucleic acids associated with HAV and not infectivity. Some free viral RNA, which is not infectious, could be found around the sample.

A study conducted by Rzeżutka et al. (2006) developed a method for the detection of HAV in soft fruits (raspberries and strawberries). Raspberries and strawberries bought from a local supplier were inoculated with HAV, and then a sodium bicarbonate mixture was added and the mixture was centrifuged. The supernatant was extracted, the pectin was removed with pectinase, and the mixture was centrifuged again. The supernatant was decanted and ultracentrifuged. HAV RNA was extracted from the pellet and was examined with RT-PCR. The estimated sensitivity of this method was 250 RT-PCR units HAV per sample assuming 100% efficiency; however, the lowest reading was 10000 RT-PCR units per sample of strawberries, and 1000 RT-PCR units in raspberries; this is a recovery range of 2.5% to 25%. The authors suggested that this method of extraction was suitable for recovering HAV from soft fruits that can be used in the field for outbreak studies.

1.19 Aichi Virus (AiV)

Aichi virus was first detected in 1989 as being responsible for oyster-associated non-bacterial gastroenteritis in patients in Aichi, Japan (Yamashita et al.

1991). It is a member of the *Picornaviridae* family in the *Kobivirus* genus with three distinct genotypes that have been identified (A, B, and C) (Yamashita et al. 1998, Yamashita et al. 2001). Few studies have been conducted on AiV and its impact on human health. Most AiV studies pertain to oysters and other shellfish outbreaks in Japan, Germany, and France (Sdiri-Loulizi et al. 2009); however, reports of AiV infections are rare, leading to limited knowledge of the epidemiological properties of AiV in humans.

Small round viruses (SRV), including Norwalk-like agents, caliciviruses, and astroviruses, have been greatly studied regarding their pathogenic potential (Dolin et al. 1987). Although caliciviruses and astroviruses had been propagated in cell lines with the addition of trypsin, it was almost impossible to isolate cytopathic human SRV in cell cultures. In a study conducted by Yamashita et al. (1991), cytopathic SRV were first isolated with BS-C-1 cells from patients with gastroenteritis associated with a non-bacterial oyster-associated outbreak. Stool samples were taken two to five days after the onset of symptoms from 12 cases in this outbreak in Aichi Prefecture in 1989; serum samples were taken from 15 cases, as well as from 4 cases that were involved in outbreaks in 1987 and 1988. The stool samples were tested for SRV by electron microscopy and viral isolation was attempted using BS-C-1 cells. The serum samples were tested for antibody to SRV by immunoelectron microscopy. Isolates were also inoculated into Vero, HeLa, HEL, and RD cells to examine the cytopathic effect. Cytopathic agents were isolated in 25% of the samples taken, and examined in BS-C-1 cells, where a mild cytopathic effect appeared 1 – 3 weeks following inoculation. After the second passage, a cytopathic effect was seen 3 – 4 days after

inoculation. The isolates were successfully titrated in Vero cells, and did not result in cell damage in HeLa, HEL, or RD.

Based on these results, it was believed that the isolates had an RNA genome (Yamashita et al. 1991). Further testing found the isolates to be stable in treatments with chloroform and at pH 3.5, as well as surviving a treatment of 50°C for 30 minutes, but not at 60°C for the same period. The isolate was roughly 30 nm in diameter, and has a star-shaped configuration that resembles astroviruses; however, although the isolates were biophysically and biochemically identical to enteroviruses, the isolates could not be serologically classified with the available 67 types that had been classified at the time. Upon closer polypeptide investigation, it was found that the polypeptide patterns in the isolates differed from those of enteroviruses. With SDS-PAGE analyses, it was found that the isolates had bands at 42, 28, 27, and 22 kDa, and running Western blotting found a major structural protein at 42 kDa. Astroviruses have major structural proteins of 16 – 45 kDa, leading Yamashita et al. (1991) to believe that the isolates were astro-like viruses. This was the first recorded isolation and characterization of Aichi virus.

The number of gastroenteritis cases associated with eating raw oysters in Japan increased from 9 to 19 between 1987 and 1989, and occur in the winter months (Yamashita et al. 1993). These outbreaks were generally attributed to small round viruses (SRV), mostly Noroviruses, caliciviruses, and astroviruses; however, as the sensitivity of testing methodologies improved, it was found that some cases have SRVs that could not be classified as Norwalk-like virus, calicivirus, or astrovirus. When AiV was isolated with BS-C-1 cells from gastroenteritis cases, these unclassified cases were pinpointed to the new virus. Aichi virus has a similar

ultrastructure to astrovirus, but is different from other enteric viruses in the biophysical, biochemical, and immunological properties. In the previous study by Yamashita et al. (1991), AiV was identified, but the reactivity of the new strain with an astrovirus by immunological methods was not investigated. In the study following, differences between AiV and astrovirus were determined via ELISA, as well as developing a monoclonal antibody against AiV for detection in stool samples.

In this study, AiV was isolated from a patient with gastroenteritis, grown in Vero cells, and guinea pig immune serum was prepared (Yamashita et al. 1993). Sixty-six types of enteroviruses, three types of reoviruses, and fourteen types of adenoviruses were obtained from the Tokyo NIH, and were grown accordingly. In addition, stool samples containing caliciviruses, astroviruses, rotaviruses, HAV, and Norwalk viruses were obtained. Adult stool specimens were collected 2 to 5 days following the onset of symptoms from 69 cases, and child stool samples were collected from 397 cases involved in 9 outbreaks of oyster-associated gastroenteritis. Extracts from the samples were grown in BS-C-1 and Vero cells. RNA extraction from all enteroviruses and AiV were performed and analyzed via RT-PCR.

In addition, paired serum samples were taken from 59 of the adult cases to test for seroconversion; acute-phase serum samples were collected 2 – 5 days following the onset of symptoms, and convalescent-phase serum samples were collected two weeks following the collection of the acute-phase serum samples (Yamashita et al. 1993). Monoclonal antibodies against AiV were prepared by injecting six-week-old mice with AiV and with purified antigen a month later as a booster. Lymphocyte hybridomas were prepared four days following the second injection by fusing spleen and NS-1 myeloma cells and then checking for secretion of

AiV antibodies. Hybridomas found the secrete AiV antibodies were injected into mice, and the immunoglobulin class was determined with anti-mouse IgM, IgG1, IgG2a, and IgG3 serum. A sandwich ELISA was then used to detect AiV antigen.

The sensitivity of the PCR was determined by attempting to detect the nucleic acids of all 66 serotypes of enteroviruses; 64 of the 66 enteroviruses were successfully amplified, but AiV was not detected (Yamashita et al. 1993). The hybridoma used in the ELISA produced an antibody reaction with AiV, and was labeled clone Ai/2 with immunoglobulin subclass G2b. Of the 578 stool samples tested for this antigen, 14 were positive. The ELISA showed that antibody reactivity did not occur with any non-Aichi strains of enteric viruses.

Although AiV is biophysically and biochemically identical to enteroviruses, it is serologically untypable with regard to the previously reported 66 types of these viruses (Yamashita et al. 1993). Additionally, heating AiV at 50°C for 30 minutes caused changes in structural proteins different from those observed for enteroviruses. The RNA was distinct from other enteroviruses based on the PCR results. Upon examination of the ultrastructure, it was believed that AiV could be in the astrovirus family; however, AiV can be cultured without trypsin, whereas astroviruses need trypsin to grow in cells. The ELISA also showed that the AiV antigen does not react in the ELISAs for the 66 types of enteroviruses, Norwalk virus, calicivirus, or astrovirus. Based on these results, Yamashita et al. (1993) concluded that AiV is a new type of SRV, or potentially a new serotype of astrovirus.

Yamashita et al. (1991, 1993) were able to identify the structure of purified Aichi virus to be small and round-structured, and to grow it in cultured cells. The virus was resistant to treatments with chloroform and pH changes; however,

enterovirus antisera did not inactivate the AiV. This led Yamashita et al. (1998) to perform molecular cloning and complete nucleotide sequence analysis to sequence the AiV genome. Aichi virus was isolated in BS-C-1 cells, purified by CsCl and sucrose density gradient centrifugation, and then grown in Vero cells. The RNA was extracted using proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. It was found that AiV is a single-stranded (+)RNA virus, consisting of 8251 nucleotides, excluding the poly-A tail. There is an open reading frame with 7302 nucleotides that encodes a polyprotein precursor of 2,433 amino acids. There was also a 5' nontranslated region with 712 bases, and a 3' nontranslated region with 240 bases, followed by a poly-A tail. The base composition was 19.5% adenine, 21.1% guanine, 37.8% cytosine, and 21.6% uracil. The AiV genome, VPg-5' nontranslated region–leader protein–structural proteins– nonstructural proteins–3' nontranslated region–poly-A tail, is analogous to other picornaviruses. In addition, the VP0-VP3 and VP3-VP1 cleavage sites were determined to be Q-H and Q-T (respectively), and cleavage sites Q-G, Q-A, and Q-S were found to cleave P2 and P3 polyproteins, which are similar to that of picornaviruses. These findings also showed that AiV is genetically different from the other 6 known picornaviruses, suggesting that AiV is a new genus of the family *Picornaviridae*, in the new *Kobuvirus* genus.

In another study conducted by Yamashita et al. (2000), AiV were isolated in Vero cells from seven cases in five separate gastroenteritis outbreaks in Japan, five Japanese citizens returning from Southeast Asian countries, and five children in Pakistan. Using the sequence derived for AiV (Yamashita et al. 1998), an RT-PCR method for the detection of AiV was developed; this method was able to detect the differences (if any) between AiV found in Japan compared with other countries. RNA

sequences were collected for 519 bases for each sample at the C terminus of 3C and the N terminus of 3D. A 90% homology was found between the samples, which were further divided into genotypes A and B. Genotype A was isolated from four cases in Japan and one case that had traveled to Southeast Asia. Genotype B was found in other outbreak cases in Japan and four cases that had traveled to Pakistan. The sequences found for these isolates allowed for the development of a primer pair as well as a biotin-labeled probe that can be used for AiV RNA amplification. This allowed for RT-PCR to be a useful tool in the detection of AiV, as well as building a library of PCR products that can be used as a reference to identify the strain present in an outbreak.

1.19.1 Prevalence

To date, little is known of the incidence of AiV infection in humans worldwide (Ribes et al. 2010). Aichi virus has been isolated in patients in the United States, Pakistan, Japan, Southeast Asia, Bangladesh, Thailand, Vietnam, Brazil, Germany, France, Tunisia, Hungary, and Finland. Studies of seroprevalence have showed a high rate of antibodies to AiV. The seroprevalence of antibodies to AiV in Valencia, Spain was examined between 2007 and 2008. Three hundred sixty-four serum samples from healthy individuals were randomly collected. Viral antigen was partially purified from infected cells prepared in the lab, and the presence of antibodies against AiV was determined via ELISA. Additionally, 23 serum samples were inactivated, serially diluted, and incubated in the presence of 50 – 100 immunoperoxidase-stained focus-forming units of AiV. The mixtures were grown in cell culture, and then the viral inocula were removed and viral antigens were detected by an immunoperoxidase assay.

Using an indirect IgG ELISA, the seroprevalence of antibodies against AiV were analyzed in the 364 serum samples (Ribes et al. 2010). Seventy percent of these samples were positive for antibodies to AiV. When broken down by age, it was found that 83% of the samples were from children ranging from 0 to 2 years old. Seroprevalence increased from 41% in ages 2 to 5 to 49% in ages 5 to 9. Cases aged 10 to 19 had 63% seroprevalence, which increased to 86% in the 20 to 24 age group. Seroprevalence was 95% in the 25 to 39 age range, and almost 100% in individuals over 40. The levels of antibodies to AiV were found to increase significantly with age, and a relationship was found between ELISA and seroneutralization.

Overall, the Spanish population in this study had a high prevalence (70%) of antibodies against AiV; studies conducted in Germany and Japan have also shown similar results (76% and 55%, respectively) (Ribes et al. 2010). Based on the results of this study, it is believed that the seroprevalence of antibodies to AiV changes geographically. Studies conducted in Japan and France have shown a plateau effect, with roughly 84% positive sera between 30 and 35 years (Yamashita et al. 1993, Goyer et al. 2008). In Germany, 86% positivity was found by 15 years, whereas in Spain, 85% positivity was found at 20 years (Oh et al. 2006, Ribes et al. 2010). These studies all suggested that younger individuals have an overall lower prevalence of antibodies, which increases with age. It is believed that the increase is a direct result of multiple infections, most of which are most likely asymptomatic.

Goyer et al. (2008) examined the prevalence of AiV in France using a serological study of 972 samples, which were divided into groups according to age. Seroprevalence ranged from 25% (7 months to 9 years) to 85% in cases over 30. This study also showed a plateau effect after 30 years which is consistent with previous

studies. The ELISA tests showed significant differences among age groups with lower antibody levels at young ages; this study also concluded that reinfections result in increased levels of antibodies as age increases. In addition, it is believed AiV infections that are generally mild, do not require medical attention, and may only be considered pathogenic when paired with norovirus or enteric viruses.

1.19.2 Outbreaks Involving AiV and Other Enteric Viruses

Gastroenteritis is a major cause of morbidity and mortality in developing countries with rotaviruses, noroviruses, sapoviruses, astroviruses, adenoviruses, and most recently AiV associated with illnesses (Sditi-Loulizi et al. 2008). Aichi viruses are most commonly associated with oyster consumption. In Tunisia, a Mediterranean country, rotaviruses, astroviruses, and adenoviruses have been found to contribute to childhood diarrhea. In this study, the contribution of noroviruses and AiV were studied over a two-year period (January 2003 – May 2005) in children that were hospitalized in Tunisia. Six hundred thirty-two children under the age of 12 were included in this study; these children all suffered from acute gastroenteritis, which was defined as multiple soft or liquid stools and vomiting in a 24-hour period, accompanied by abdominal pain and fever. Samples were screened out of bacterial agents and then analyzed for enteric viruses; 308 samples were collected by the end of the study. Samples were subjected to enzyme immunoassay and RT-PCR, and genotyping was performed by direct sequencing of the PCR products with the same primers used for amplification.

Of the 632 children in this study, 43.7% had a positive stool sample for an enteric virus (Sditi-Loulizi et al. 2008). Of these positive samples, 84.8% were infected by one virus, 13.4% were infected by two viruses, and 1.9% were infected by

three different viruses. In addition, 22.5, 17.4, 4.1, 3.5, 2.7, and 1% of the samples were positive for rotaviruses, noroviruses, astroviruses, AiV, adenoviruses, and sapoviruses, respectively. Of the cases infected with one virus, rotaviruses and noroviruses were the most common, with only 6.8% of the cases infected with AiV. It was surprising to find AiV infections not associated with oysters in the children in this study, suggesting that oysters may not be the only vector for transmission of AiV. This was the first study to investigate the role of currently recognized viruses, such as noroviruses, sapoviruses, and Aichi viruses, in the infections of children (Sditi-Loulizi et al. 2008).

In a study conducted by Sditi-Loulizi et al. (2009), stool samples were collected from 788 Tunisian children suffering from diarrhea that were under 12 years old. This study was expanded to over four years (January 2003 – April 2007), and involved 408 children that had been hospitalized and 380 children from outpatient clinics. All of the samples were negative for bacterial pathogens and parasites. Aichi virus was detected by RT-PCR, using a primer pair to amplify a 519-bp fragment at the 3CD junction; the PCR fragments were then used for genotyping. A little over four percent of the samples tested positive for AiV only, and 6.2% of the samples were infected with AiV and either rotavirus or astrovirus. The AiV strains found in this study were all genotype A, and were divided into groups H1, H2, or H3 based on the slight variations in nucleotide sequences found. It is believed that AiV genotypes may be geographically separated based on results from other studies. Only genotype A has been found in Germany and France, and is the most dominant genotype in Japan. On the other hand, genotype B has been isolated from cases in Pakistan, Bangladesh, Brazil, and Malaysia. It is believed that genotype C is predominant in most of Africa,

although data is lacking to support this hypothesis. This study further showed that AiV may be a causative agent of pediatric diarrhea, and may not only be associated with shellfish consumption.

In February 2006, an outbreak in France was associated with oyster consumption. In this outbreak, stool samples identified as many as seven different strains in one sample. The purpose of this particular study was to determine if shellfish was associated with the outbreak for the presence of enteric viruses and to report the presence of AiV in Europe for the first time (LeGuyader et al. 2008). It was believed that the lagoon in which the oysters were harvested was contaminated, and that prevention measures, such as prolonged depuration time to drop *E. coli* counts, were not enough to inactivate human enteric viruses.

Cases involved in this outbreak were identified by medical doctors and sanitary services. These cases were given a questionnaire concerning foods consumed and the onset of symptoms. Twelve stool samples were collected from the cases. Group A rotaviruses, astroviruses, and adenoviruses were detected via enzyme immunoassay with group-specific antibodies as necessary, and the results were confirmed with RT-PCR; the presence of enteric viruses was determined by nucleic acid extraction (LeGuyader et al. 2008). Noroviruses and sapoviruses were detected by multiple RT-PCRs that amplified regions on the RNA-dependent RNA polymerase and capsid genes. HAV was identified by amplification of a VP1 gene fragment, and enteroviruses were identified by the amplification of the 5' untranslated region. Aichi virus was detected by amplification of a 519-bp fragment. In addition, 66 samples of oysters were collected, four of which were directly linked to human consumption. Viral nucleic acids were extracted and screened by real time RT-PCR. The viruses

that were detected were typed by sequencing after amplification; amplicons from virus-positive samples were purified and sequenced, and then analyzed through the European Foodborne Viruses Database.

At the end of this outbreak 205 cases were reported, with two hospitalized. Fifty-eight percent of these cases had purchased oysters at a market and consumed at home, while thirty-five percent of these cases consumed oysters at a restaurant (LeGuyader et al. 2008). The study showed that consumption of oysters increased the risk of illness 4.5 times than the counterparts that did not consume oysters. Of the twelve stool samples analyzed, one sample did not contain any viral pathogens, and all were negative for adenovirus, sapovirus, and HAV. Seventy-five percent of the samples were positive for norovirus and half were positive for AiV and enteroviruses. Eight of these samples had more than one pathogenic virus, and one sample tested positive for seven different viruses. Of the shellfish samples, two samples were positive for AiV, adenovirus, norovirus, and rotavirus. Sixty-two samples from the same lagoon where the oysters were harvested were collected; seven of these samples were negative for any enteric virus, 16 were contaminated by one enteric virus, and 39 were contaminated by at least two different viruses. Adenoviruses were detected in a large number of the samples, rotaviruses were detected in 15 samples, noroviruses were detected in 33 samples, and 22 samples contained noroviruses. Aichi virus was detected in five samples; the sequence analyses found that AiV sequences were identical between oyster and stool samples.

In the winter of 2006, gastroenteritis cases were frequent which may have been a result of adverse weather conditions (LeGuyader et al. 2008). In one week, heavy rains were constant which was found to increase the levels of *E. coli* in the

harvesting lagoon. Although depuration methods were undertaken for two weeks prior to harvest to decrease bacterial levels, viral concentrations did not diminish. In previous outbreaks containing multiple strains of pathogenic viruses, noroviruses were generally the culprit; these outbreaks also were associated with shellfish. This outbreak was different, however, in the diversity of viruses found in the samples. This made diagnostic measures difficult due to the fact that clinical signs could not be used to discriminate between the viruses. In addition, this is the first study in which AiV was detected in shellfish in Europe.

In a study conducted by Ambert-Balay et al. (2008), the prevalence of AiV strains in sporadic outbreaks (children hospitalized for acute gastroenteritis from 2001 – 2004) and epidemic outbreaks (cases of gastroenteritis outbreaks from January 2006 – April 2007) in France were examined. Stool samples from 457 children of sporadic cases and 566 adults involved in outbreaks were collected and found to be bacteria-free. Viral nucleic acid was extracted from the stool samples and screened for the presence of group A rotaviruses, astroviruses, adenoviruses, caliciviruses, and AiV by enzyme immunoassay (using group-specific monoclonal antibodies) and RT-PCR. The RT-PCR products were sequenced and were determined at least twice in both directions.

Of the 457 child stool samples, four were positive for AiV RNA; two of these samples had AiV and rotavirus, and the other two samples were only infected with AiV. The remaining samples were infected with group A rotaviruses (52.3%), noroviruses (12%), enteric adenoviruses (3.5%), astroviruses (1.5%), and sapoviruses (0.4%) (Ambert-Balay et al. 2008). Of the 566 samples associated with outbreaks, nine were positive for AiV; four of these were only infected with AiV, and the other

five were combined with noroviruses. The remaining samples were infected with noroviruses (19.4%), rotaviruses (2.1%), astroviruses (0.88%), enteroviruses (0.88%), and sapoviruses (0.18%). Many of these infections were mixed infections. The cases related to AiV were associated with oysters, and were found to be classified as genotype A, and were found to have 95% homology in nucleotides and 99% homology in amino acids. The results of this study led Ambert-Balay et al.(2008) to hypothesize that AiV is a causative agent of gastroenteritis.

It is believed that AiV genotypes differ based on geographical locations. In the above study, the AiV genotype found was genotype A, whereas in the following study, the AiV is genotype B. In a study conducted by Alcalá et al. (2010), the distribution and persistence of sewage-borne viral pathogens in highly contaminated waters were examined. Prior to this study, it was unknown if AiV circulated throughout the population of Venezuela, which may be attributed to undiagnosed cases of gastroenteritis. Eleven samples were collected from an urban stream (Guaira River) with samplings taken twice a month from October 2007 – February 2008; this particular river has been cited in other papers because of its constant high levels of pollution (Betancourt et al. 2010, Rodríguez-Díaz et al. 2009). Detection and characterization of AiV were completed by RT-PCR and nested PCR, using primers amplifying a 519bp fragment between the 3CD region and the N terminus. PCR fragments were purified and sequenced. Aichi virus was detected by RT-PCR in 5 of the 11 samples collected, and all found to be genotype B. In addition, the nucleotide sequences were found to be similar to other AiV strains throughout the region, suggesting circulation of closely related strains between regions (Alcalá et al. 2010).

1.19.3 Attachment Studies

Attachment of pathogens to leafy greens is an important contribution to foodborne outbreaks. The factors that affect this pathogen attachment (physical, chemical, and biological) can provide insight into preventative measures controlling contamination. Studies have shown that viruses attach differently to lettuce surfaces based on their individual isoelectric points (pI), which affect the net surface charge of the virus (Vega et al. 2005).

In a study conducted by Vega et al. (2005), it was hypothesized that the pI of a virus affects the attachment to the surface of lettuce. Bacteriophages MS2 and ϕ phage, echovirus 11, and FCV were used in this study. Butterhead lettuce was cut into equal sections, and submerged in citric phosphate buffer or sodium phosphate buffer. The buffers were titrated into a pH range that encompassed the pI values of the four viruses. The viruses were added to the samples, allowed to adsorb for 30 minutes, and then measured.

Adsorption of the MS2 bacteriophage changed the most as a function of pH, with maximum adsorption at pH 3.0. At pH 8.0, most viruses were unattached; FCV exhibited maximum attachment at this pH. Echovirus 11 had the greatest attachment of the four viruses, and ϕ exhibited the least amount of attachment. Overall, as pH increased from 5.0 – 8.0, attachment increased. The results of this study suggest that these viruses do not use specific cell surface receptors to attach to the lettuce; it is believed that electrostatic forces, hydrophobic forces, or Van der Waals forces play a role in viral attachment. The DLVO theory places attachment of viruses to surfaces on Van der Waals (attractive) and electrostatic (repulsive) forces; factors that decrease or increase the electrostatic component will inversely effect the

Van der Waals forces, which will affect the overall adsorption (Vega et al. 2005). The DLVO theory also states that attachment decreases with increasing pH as a result of increased repulsion by negatively charged virus particles, thereby increasing above the viral pI; however, only the MS2 bacteriophage acted according to the DLVO theory. It was concluded that the pI of the virus is not the only factor in virus attachment.

In another study conducted by Vega et al. (2008), the role of hydrophobic and electrostatic interactions in the attachment of enteric viruses to leafy greens was examined, as well as examining the effects of removing these forces. By understanding these forces, it may be possible to develop a wash treatment to effectively remove enteric viruses attached to the surface of the leafy greens. In this study, four different viruses were employed: MS2 and ϕ , both of which are bacteriophages used as fecal viral indicators and as surrogates, FCV, a norovirus surrogate, and echovirus 11, a human pathogen. In addition, these four viruses have similar physio-chemical characteristics. Butterhead lettuce was used in this study because of previous studies showing that enteric viruses use electrostatic forces to attach to this particular vegetable. The lettuce leaves were cut into equal sections, submerged in citric phosphate buffer or sodium phosphate buffer with virus, and then examined to determine if electrostatic or hydrophobic forces were involved in virus attachment. In order to determine what forces play a role in viral attachment, the electrostatic, hydrophobic, and electrostatic and hydrophobic forces were inhibited by the addition of NaCl, Tween 80, or NaCl and Tween 80, respectively.

Attachment of echovirus 11 increased in the presence of NaCl and decreased adsorption; in the presence of Tween 80, attachment increased while adsorption remained relatively constant. When both NaCl and Tween 80 were

present, echovirus 11 adsorbed only at low levels. In the presence of NaCl, FCV adsorption remained at 0, and remained constant in the presence of Tween 80; when both NaCl and Tween 80 were present, no adsorption was measured. The MS2 bacteriophage increased adsorption and decreased attachment in the presence of NaCl, decreased adsorption in the presence of Tween 80, and was not affected by the combination of NaCl and Tween 80. The ϕ bacteriophage was not affected by any of the treatments. The results of this study led to the hypothesis that Van der Waals forces play a role in virus adsorption; NaCl produces highly charged ions in solution, compressing the Gouy layer, rendering electrostatic forces useless (Vega et al. 2008). Since Tween 80 had very little effect on the viruses, the Van der Waals forces becomes a more likely explanation.

1.20 Bacterial Work

It is estimated that over 90% of confirmed foodborne human illness cases and deaths reported to the CDC are linked to bacteria (Bean et al. 1990). Most of these infections occur as a result of consumption of contaminated foods, although slaughterhouse workers are also potentially exposed to brucellosis, psittacosis, and tuberculosis (Buzby et al. 1996). Between 1986 and 1995, over 800 foodborne outbreaks including over 26,000 cases and 20 deaths were reported in Taiwan (Pan et al. 1997). Data from foodborne outbreaks have been collected by the Department of Health since 1981; however, data points such as epidemiologic data, number of cases, etiology, sources of contaminated food, and place of preparation were only recorded after 1986 (Choiu and Chen 1991). It was found that approximately 80% of the foodborne outbreaks occurred between April and October with peaks in May and October. Separate peaks for *V. parahaemolyticus* and *B. cereus* outbreaks were found

in June and September. The total number of outbreaks was found to decrease in August, which was assumed to be because of a lack of person-to-person contact due to summer vacations and religious holidays. Between 1986 and 1993, the numbers of outbreaks varied from year to year, but between 1994 and 1995, there was a significant increase in foodborne outbreaks. It was estimated that 65% of the reported foodborne outbreaks were due to bacterial agents: *V. parahaemolyticus*, *S. aureus*, and *B. cereus* accounted for most of the bacterial outbreaks (35.5%, 30.5%, and 18.7%, respectively) (Pan et al. 1997). In 1990, the number of outbreaks due to *V. parahaemolyticus* significantly increased in Taiwan with serovar K8 isolated the most frequently (Pan et al. 1996). Outbreaks due to *V. parahaemolyticus* have also been recorded in the United States (Bean and Griffin 1990). In addition, enterotoxin A-producing strains of *S. aureus* were the most frequently isolated during outbreaks.

Pathogenic *E. coli* and *Salmonella* accounted for 6.5% and 5.6% of the outbreaks, respectively, with *C. botulinum* causing 1.8% of the outbreaks (Pan et al. 1997); however, pathogenic *E. coli* was examined only after other foodborne pathogens were ruled out which may result in an underestimation of the number of outbreaks involving pathogenic *E. coli*. The *Salmonella* serovars found during this study varied, with *S. Typhimurium* and *S. Virchow* being the most prevalent. Outbreaks of *C. botulinum* were not found after 1991. Although outbreaks due to *Plesiomonas shigelloides* and *V. cholerae* non-O1 are prevalent in Asian countries, less than 1.4% of the outbreaks in Taiwan were caused by these microorganisms. Most of the outbreaks in this study were due to seafood (Pan et al. 1997).

1.20.1 *Escherichia coli*

Escherichia coli a gram-negative straight rod that uses peritrichous flagella for motility, or are nonmotile (Hayashi et al. 2006); cells are generally 2 μm long and 0.5 μm in diameter, with a volume of approximately 0.6–0.7 μL^3 (Kubitschek 1990). They are facultatively anaerobic chemoorganotrophs that have both respiratory and fermentative metabolism. While nonpathogenic *E. coli* exist in the lower gut of animals, pathogenic *E. coli* strains can infect the enteric, urinary, pulmonary, and nervous systems of the hosts. *E. coli* can also survive outside of the body, providing an excellent indicator of fecal contamination (Reid et al. 2001). These bacteria also grow quickly and easily, making *E. coli* one of the best studied prokaryotic organisms important in biotechnology. The optimal growth temperature for *E. coli* is 37°C, and can still be found to multiply at 49°C (Fotadar et al. 2005). *E. coli* and related bacteria transfer DNA via bacterial conjugation, transduction, and transformation, all of which spread genetic material throughout the bacterial population. The gene encoding shiga toxin from *Shigella* to *E. coli* O157:H7 is believed to be transferred by these processes to a bacteriophage (Brüet al. 2004).

The most common illness associated with transmission of foodborne *E. coli* is gastroenteritis. The strains generally associated with these illnesses are O157:H7, O121, and O104:H21 (Tauschek et al. 2002). The O157:H7 strain has also been found to cause the life-threatening illnesses HUS, and was the cause of an outbreak involving fresh spinach in the United States in 2006. Bacterial contamination of foods can occur anywhere along the farm to fork continuum, with dairy and beef cattle primary carriers of *E. coli* O157:H7, usually carrying it asymptotically (Bach et al. 2002). Foods commonly associated with *E. coli*

contamination include raw ground beef, raw seed sprouts or spinach, raw milk, unpasteurized juice, and unpasteurized cheese (Heaton and Jones 2008).

E. coli O157:H7 infections have recently been attributed to fresh lettuce and spinach in the United States (Brandl 2008). Unfortunately, the source of these outbreaks are generally not pinpointed to pre- or post-harvest conditions, as *E. coli* O157:H7 can survive on the surface of leafy greens in the field as well as multiply in the phyllosphere of lettuce in warm and wet conditions (Brandl and Amundson 2008). Harvesting and post-harvest operations of leafy greens damage plant tissue. *E. coli* O157:H7 will attach to the cut edges of lettuce, as well as trichomes, stomata, and cracks in the cuticle (Seo and Frank 1999). Additional studies on modified atmosphere packaging have shown that *E. coli* O157:H7 can multiply on cut and damaged leaves for extended periods of time at temperatures ranging from 10°C to 15°C, and is promoted when pretreated with warm chlorinated water (Delaquis et al. 2002, Li et al. 2001). Similar data have been collected with *S. enterica* and *Shigella sonnei* (Wu et al. 2000).

In a study conducted by Brandl (2008), the contamination risk associated with minimally processed leafy greens and the levels of growth of *E. coli* O157:H7 in damaged areas. Romaine lettuce leaves were grown and the leaves were harvested. The stems were cut into sections and inoculated, while the leaves were submerged in an inoculum suspension and were then left intact, bruised, cut into pieces, or shredded. After 2 and 4 hours, it was found that the levels of *E. coli* O157:H7 increased 5.6- and 11.1-fold, while after 22 hours, the levels of *E. coli* increased 20,091-fold. These data suggest that a substrate is present on cut stems that promote the growth of *E. coli* O157:H7. Within 4 hours of inoculation to the leaves, the bacterial levels increased

3.99-fold on bruised samples, 4.54-fold on cut leaves, and 11.05-fold on the shredded leaves, while increasing by a mere 1.95-fold on intact leaves. These data suggest that the colonization by *E. coli* is correlated to the extent of leaf damage; this may be due to enhanced attachment of the pathogen to substrates located at the cut areas of the leaf, as was seen with the cut stems. In addition, lettuce is generally stored in bins in the field prior to transport to a processing facility and may be exposed to elevated temperatures and humidity, which promotes bacterial growth.

Data suggesting that bacterial pathogens can increase as much as 11-fold within 4 hours is important to take into consideration when developing new GAPs and HACCP protocols (Brandl 2008). Recent *E. coli* O157:H7 outbreaks attributed to leafy greens and their ability of rapid multiplication in damaged areas of leaves over a short period should be taken into consideration in risk assessment.

1.20.2 *Salmonella* spp.

Salmonella spp. are gram-negative, non-sporeforming, facultative intracellular bacteria that can enter the body through nasal, ocular, oral, or intestinal mucous membranes (Simmons 2002). These rod-shaped bacteria are generally 0.7 µm to 1.5 µm in diameter, 2 µm to 5 µm in length, and are chemoorganotrophs. Most species of *Salmonella* exist in motile phase I or nonmotile phase II (Clark and Barret 1987). In fact, *Salmonella* are closely related to *Escherichia* and are found in humans and warm- and cold-blooded animals. Infection generally occurs via the fecal-oral route, transmitted through contaminated livestock and rodents; contamination can result in typhoid fever, paratyphoid fever, and salmonellosis (Ryan and Ray 2004). Estimates suggest that 40% of animal byproducts (fish meal, bone meal, feather meal)

in the United States are contaminated, as well as plant proteins such as soybean and cottonseed.

Non-typhoidal salmonellosis is becoming increasingly prevalent in European countries and the United States (Scuderi et al. 1996). In the United States, the rates of salmonellosis increased from 7 per 100,000 people in 1955 to 17 per 100,000 people in 1994, with a peak of 27 per 100,000 people in 1986 (CDC 1994). In the United Kingdom, salmonellosis rates increased from 10,761 cases in 1980 to 22,627 cases in 1991, to 23,367 cases in 1994 (Stockett et al. 1993, CDSC 1994). Gastroenteritis due to *S. enteritidis* and *S. Typhimurium* were the second most common infections in the United Kingdom in 1989, with 54,268 total cases reported (Scuderi et al. 1996). Between 1985 and 1992, 430 cases of *S. enteritidis* were reported (CDC 1993). In Italy, *Salmonella* spp. are reported via the Laboratory Surveillance System of Enteropathogenic Bacteria, which divides the bacteria into human, animal, and foodborne groups (Scuderi et al. 1996). In addition to this surveillance system, the National Infectious Disease Reporting System was established in 1991, mandating the reporting of potential outbreak scenarios. Between 1987 and 1993, the incidences of *Salmonella* spp. rose from 5,759 to 13,600. In Germany, *Salmonella* infections are required to be reported; between 1990 and 2005, cases of *Salmonella* in Germany went from 200,000 cases to 50,000 (Lynch et al. 2006). Estimates place worldwide salmonellosis infections at 16 million cases per year.

Studies have shown that some strains of *Salmonella* can survive outside of the body for weeks and have been found viable in dried feces after 2.5 years (Simmons 2002). *Salmonella* are not destroyed by freezing, but are damaged by

ultraviolet radiation and high temperatures. In a study conducted by Scuderi et al. (1996), 1,699 foodborne outbreaks between 1991 and 1994 were investigated. The outbreaks were reported to the National Institute of Health of Italy in Rome, and the most common (81%) was caused by *Salmonella* spp. *Salmonella* Enteritidis (34%) and non-serotyped group D *Salmonella* (33%) were the most common during this time frame. The source of 69% of the outbreaks was pinpointed; it was found that eggs accounted for 77% of these outbreaks. The *Salmonella* strains were isolated and the phenotypic and genotypic characteristics were examined. *S. Enteritidis* was involved in 50 outbreaks, *S. Typhimurium* in 3 outbreaks, and *S. Hadar* in one outbreak. The *S. Enteritidis* outbreaks were 64–68 % phage type 4, followed by phage type 1 in 14–18% of the outbreaks.

1.21 Conclusion

One hundred years ago, typhoid fever, tuberculosis, and cholera were common foodborne diseases; however, improvements in food safety, including pasteurization, safer canning methodologies, and disinfection of water supplies have lowered the incidences of these diseases. Today newly recognized microbes are more prevalent in foodborne disease transmission, due to improved technologies that can now identify pathogens that were previously unrecognized.

It is important to keep food safe from the farm to the fork for the prevention of foodborne outbreaks. Improving technologies and the increase of knowledge allows public health groups, industry, regulatory agencies, and academia to acknowledge and play a role in making the food supply safer for consumers. Improvement of agricultural practices to limit pathogen transmission is key, along with developing and recognizing critical control points where contamination can be

prevented, limited, or eliminated. Similarly, consumers also play a role in properly washing and preparing food prior to consumption. In the end, it is up to the consumer to demand a safe food supply; up to industry to produce it; up to researchers to develop better ways of doing so; and up to government to see that it happens, to make sure it works and to identify problems still in need of solutions.

Chapter 2

THE EFFECTS OF BROAD-SPECTRUM FERTILIZERS ON HUMAN PICORNAVIRUSES

2.1 Abstract

In this study, we set out to determine the sensitivity of two environmentally-transmitted human picornaviruses, hepatitis A virus (HAV), and Aichi virus (AiV) to commonly used plant fertilizers. While HAV and AiV are similar in morphology, they differ in response to common inactivation measures. Virus infectivity was first assessed using a traditional biocide testing method. Six broad spectrum fertilizers were evaluated. Fertilizers were mixed according to manufacturer's instructions with sterile water. Viruses were dried onto glass coverslips or suspended in liquid, treated with the fertilizer, and incubated. Virus was recovered from the coverslips and infectivity assessed by TCID₅₀.

Hepatitis A virus was found to have up to 3-log reduction in the presence of fertilizers while attached to a solid surface, and up to almost 2.5-log reduction when suspended in liquid. Aichi virus was found to have up to 2.5-log reduction when attached to a solid surface, and 2-log reduction when suspended in liquid. However, overall the viruses were found to survive treatment with the fertilizers, although the viruses were slightly more susceptible when attached to a solid surface. The results of this study suggest that fertilizers may not inactivate viruses in contaminated water.

2.2 Introduction

Contamination of ground waters and food supplies by pathogenic microorganisms is common in many areas of the United States, and public health concerns are increasingly focused on viruses. Approximately 76 million Americans are affected each year by foodborne illness, many of which are unreported and from unknown causes (Mead et al. 1999). Of the 38 million cases with known causes over 30 million are caused by viruses. An increasing percentage of these illnesses are associated with consumption of fresh produce. Outbreaks from fresh produce have reportedly increased by 295% between 1990 and 2001 (Mead et al. 1999). Non-potable water used in the farm environment may result in crop contamination (Tyrrel et al. 2006); the risks associated with water employed in the production of leafy greens and other vegetables must be identified. Fresh fruits and vegetables are commonly consumed in their raw state without processing to reduce or eliminate viral pathogens; therefore, managing the manner in which they are grown and harvested is crucial to minimize microbial contamination (Sobsey et al. 1978).

Viral contamination of food can occur from pre-harvest (i.e., from soil, irrigation water, water used to apply pesticides and fertilizers, dust, insects, land-applied manures and biosolids, and animals) to post-harvest (harvesting equipment, transport containers, animals, insects, dust) (Beuchat 2002). Protecting public health requires a sound fundamental understanding of the factors which impact the fate and transport of viruses in agricultural systems. While viruses may likely be transmitted to

plants through contaminated water; there is no evidence to show the fate of viruses when contaminated water is used in the application of pesticides and fertilizers.

Application of animal wastes and biosolids to agricultural land can disseminate pathogens in the environment. Although viruses are not the only pathogens of concern, they are much smaller in size than other enteric pathogens, and can move better through the subsurface (Ferguson et al. 2003). Viruses also tend to survive longer than bacteria and protozoa in the environment and therefore pose greater threats for groundwater and produce contamination. There is evidence that viruses can move considerable distances in specific soil layers (Yates and Yates 1988). Such findings have raised concerns about surface and ground water contamination. Generally, viruses survive longer under moist as compared to dry conditions (Yates and Yates 1988). In addition, when viruses are associated with a solid surface, they are generally protected from being inactivated (Gerba 1984; Gerba and Goyal 1981).

Fertilizers used in agriculture can be in either solid or liquid form. Although crop responses to liquid and solid fertilizers are similar, solid fertilizers are mainly used in commercial growing operations (Westfall and Hanson 1985); however, liquid fertilizer use in the United States has steadily increased over the past 25 years (Spicer 2002). Nitrogen fertilizers are the most common form of liquid fertilizers currently used with ammonium and nitrate fertilizers the most popular (Westfall and Hanson 1985). Most phosphate fertilizers are also highly water soluble.

The application of fertilizers made with contaminated or non-potable water is an area of risk for contamination of fruits and vegetables. Viruses and bacteria

may survive on crops or within the soil and then migrate to crops during the growing season (Passioura 2007). The most common fertilizers used are “N-P-K” fertilizers, which refers to the specific ratios of nitrogen, phosphorus, and potassium in each fertilizer. The different ratios are used for different points in the growing process, and are generally mixed throughout the growing season for the best crop results.

Fertilizers with high levels of nitrogen are typically used for “green growth,” which is the total biomass of the crops. Nitrogen fertilizers are generally applied to off-season cover crops, increasing the amount of available nitrogen in the soil for the main summer crops (Schmidt et al. 2009). Phosphorus is used for root growth in crops, and is added to the soil prior to planting. Triple Superphosphate is widely used in soils with phosphorus deficiencies, which is becoming a problem in industrialized countries; however, the United States is trying to outlaw the use of phosphorus in fertilizers due to increasing instances of groundwater contamination (B. Clements, personal communication, 27 August 2010). Potassium in fertilizers is used for overall strength and health. These fertilizers are not as susceptible to leaching as phosphorus, and tend to stay close to the initial point of application. In order to achieve the healthiest and most productive crops, routine testing of soils and the fertilizers used are necessary.

Each fertilizer used in this study contains several components that may play a role in microbial inactivation; more importantly, these ingredients affect the usefulness of these fertilizers. The main chemical components of each are discussed below, and shown in Table 3ab.

Urea: Urea is the most widely used nitrogen fertilizer with the highest concentration of nitrogen of any fertilizer in today's market. It is 100% water-soluble, and is mobile in the soil, moving with soil moisture until soil microorganisms initiate nitrification. This reaction produces ammonium in two to three days following application, and can occur in less than a day in warmer environments. This can cause a problem when applying urea in the summer, because volatilization of ammonia occurs at a quicker rate when surface applied. Since urea travels well throughout the soil, there is a potential risk of groundwater contamination; however, when released into water, urea will biodegrade to a moderate extent. Urea can be applied to soil as a solid or solution, and involves little explosion hazard. In addition, urea will supply a continuous, long-lasting supply of nitrogen to the growing crop.

Ammonium nitrate: Ammonium nitrate is the preferred source of nitrogen when used in combination with herbicides for "weed and feed" applications. This fertilizer is a great non-specific nitrogen fertilizer that is used early in the plant cycle, providing three forms of nitrogen (nitrate, ammoniac, and organic nitrogen). These forms of nitrogen provide both fast acting and long lasting plant food; however, this fertilizer should not be used for fall application because the cooler temperatures increase the rate of denitrification and leaching through the soil following application (up to one-half the total amount applied). In addition, this fertilizer has been found to be extremely compatible with other fertilizers and herbicides.

Ammonium sulfate: Ammonium sulfate availability has increased in recent years, and is popular in topdress application of nitrogen and sulfur. This fertilizer is

popular because it is the cheapest source of sulfur; sulfur deficiencies are becoming more prevalent in western agriculture. The nitrogen in this fertilizer is in the ammonium form, which is good material for high-pH soils (pH > 7.0) and soils with a sulfur deficiency. Ammonium sulfate is the most acidifying nitrogen fertilizer (pH 5.5-6.0), and requires addition of limestone to neutralize the acidity when applied to crops.

Calcium nitrate: Calcium nitrate is used in the fruit and vegetable industry as a quick source of nitrogen. This fertilizer has all of its nitrogen in nitrate form, which helps plants absorb other nutrients in addition to calcium. Plants require high amounts of calcium due to the composition of the cell wall. Unfortunately, calcium is generally found in soil in an unusable form, and does not transport well in plants. Since crops take up both the calcium and nitrate, there is no residue left in the roots, as is common with many other fertilizers. Unfortunately, nitrate is highly susceptible to leaching and denitrification losses immediately following application to soil, especially in clay soils.

Triple superphosphate: Triple superphosphate has a high concentration of P₂O₅ in uniform, free flowing granules, and is highly effective in eliminating phosphorous deficiencies in any soil conditions. This fertilizer is most effective when applied to the soil prior to planting. It is most often used in alfalfa and legume crops yet can still be used as a topdress throughout the year for many other crops.

Peters excel: Peters excel provides a continuous source of calcium and magnesium to soft water, enhancing the absorbance of these nutrients to crops. This fertilizer is usually used in conjunction with calcium nitrate.

Little information is available on the fate of viruses in animal waste and manure-amended soils. The available literature suggests that enteric viruses can be even more persistent in manure and manure-amended soil than bacterial pathogens (Gessel et al. 2004). Manure application rate was positively correlated with the persistence of coliphages, but they did not relate to the survival of indicator organisms. In 2006, foodborne illnesses caused by viruses accounted for 40% of the confirmed cases in the United States, with 505 cases involving caliciviruses, 5 cases of hepatitis A, and one mixed-viral case (CDC 2008).

Due to the recent attention given to foodborne disease outbreaks, especially with lettuce, there is a renewed desire to understand the origin of plant contamination by viruses. Transmission of human pathogens to plants through contaminated irrigation water and contaminated hands has been documented under both laboratory and field conditions (Stine et al. 2005). The route of viral contamination often goes unidentified for a majority of foodborne outbreaks involving fresh produce. Nearly 75% of these outbreaks are related to domestically grown produce with less than 10% from imported produce and the rest unknown. As produce consumption has increased with “Eat 5 a Day” campaigns and increased awareness of health benefits, foodborne illness associated with these foods has as well. The CDC estimates the incidence of foodborne illness attributed to fruit and vegetable consumption at 3 million cases in

the U.S. annually (Sivapalasingam et al. 2004). Viral contamination of produce has been associated with various types of fruits, leafy vegetables, and herbs. For example, as a result of on-the-farm contamination, outbreaks of hepatitis A have been associated with berries, lettuce, and green onions (Hutin et al. 1999, Rosenblum et al. 1990).

It is understood that pathogenic viruses like hepatitis A are very stable in the environment (Sobsey et al. 1988). Viruses have previously been shown to survive on the surface of vegetables for more than 2 months under suitable conditions with the potential for survival for over 2 years based on laboratory calculations within the field environment (Stine et al. 2005), outlasting the normal shelf-life of some products (Crocini et al. 2002; Kurdziel et al. 2001). Widespread transmission has been documented when commercial farms or packing houses prepare produce that is then distributed to geographically distant locations. In the 2003 hepatitis A outbreak, the Mexican producer of the green onions was found to wash produce with water from a nearby reservoir rather than with potable drinking water as required under the hygiene standards and FDA guidelines (Wheeler et al. 2005). It is likely that this water was also used for pesticide and fertilizer applications. Due to the uncertainty of the use of non-potable water for the application of fertilizers six commercially-available fertilizers were investigated for their effects on two picornaviruses.

2.3 Materials and Methods

2.3.1 Viruses and cells. Hepatitis A virus (HAV, ATCC VR-1402) and Aichi virus (AiV) strain A846/88 were kindly provided by Dr. David Kingsley, USDA, Dover, DE. These viruses were propagated and assayed on fetal rhesus monkey

kidney cells (FRhK-4 cells, ATCC CRL-1688 in Dulbecco's modified Eagle medium (DMEM, Mediatech, VA.), and Vero cells (ATCC CCL-81) in minimal essential medium (MEM, Mediatech, VA). Growth media were supplemented with 2 or 10% fetal bovine serum (FBS, Mediatech, VA), Penicillin/Streptomycin (Cambrex Bio Science, MD), and Amphotericin B (Mediatech, VA). Virus stocks were propagated and stored in media with 2 % FBS at -80°C until used.

2.3.2 Virus Quantification. Virus viability was determined using the TCID₅₀ assay (tissue culture infectious dose). Cell monolayers were grown in 96-well cell culture plates containing media with 10% FBS at 37°C with 5% CO₂. The cell monolayers were inoculated with serially diluted virus, incubated for 90 min, and the media were diluted with an equal volume of with media containing 2% FBS (Deng and Cliver, 1995). The plates were incubated for a specific amount of time (3 to 5 days for AiV, 14 to 21 days for HAV), cytopathic effects were read, and virus titer calculated according to the Reed and Meunch calculation.

2.3.3 Virus Survival in Fertilizers. Six different broad spectrum commercially-available fertilizers were evaluated (Table 3a). It is important to take into account the water solubility of the differing fertilizers.

The fertilizers were mixed according to manufacturer's instructions with water containing approximately 10⁷ virions/ml. Virus survival was tested in dry films as solid surface tests and in liquid to mimic what might happen if preparations are made in advance and then stored before being applied.

Virus in Hanks Balanced Salt Solution (HBSS) was run with each experiment as

a positive control. Changes in cytopathic effect (CPE) were measured and the virus titer determined by TCID₅₀ assay (Tissue Culture Infectious Dose). This assay takes into account loss of virus that may occur by comparing it to untreated control samples. The percent of virus recovered (baseline for sample) was determined as follows:

$$\text{baseline} = (\text{titer of recovered virus after drying}) / (\text{titer of virus at time zero}) \times 100$$

(Bidawid et al., 2004).

2.3.4 Solid Surface Tests. In order to mimic the EPA standards for disinfection, dry viral films were tested in a single test system, as previously described for the evaluation of disinfectants (Gulati et al. 2001). In brief, 11 µl of the virus suspension (10^7 virions/ml) were added to coverslips in 6-well plates, and 275 µL of fertilizer was added to each well. A control of virus in DI water was also used. The 6-well plates were kept at 20°C and 37°C away from the light for 10 min, 2 h, 24 h, 3 d, and 7 d. Following the incubation period, 725 µL of Lethen was added to each well, and the coverslips were scraped with a sterile cell scraper. This solution was added in 11 µL aliquots to the first row of a 96-well plate, along with 100 µL of 10% media. These were then serially diluted, leaving the last row as a negative control. The plates were read at 5 d for AiV and 15 d for HAV for cytopathic effect.

2.3.5 Liquid Tests. The protocol for the viral suspension was similar to that described above with virus and fertilizer combined in microcentrifuge tubes. Viruses were recovered, processed for cell culture infection, and cell culture plates were read at 5 d for AiV and 15 d for HAV for cytopathic effect.

2.3.6 Virus Interaction with Lettuce. Eleven microliters of viral suspension (10^7 TCID₅₀/ml) was added to fertilizer solution for a total of 1 ml and incubated overnight at 20°C away from light. Romaine lettuce leaves were washed and cut into 1 cm squares with a sterile scalpel and placed into wells in a 6-well plate. The virus and fertilizer mix was added to each of the wells, and the plates were kept in a humid bag (wet paper towel placed under the 6-well plate, sealed in a bag) at 4°C for three days. The liquid was removed, added to a 96-well plate, and serially diluted. The plates were read at 5 d for AiV and 15 d for HAV for cytopathic effect.

2.3.7 Bacterial Tests. For additional comparison, we used two strains of bacteria (*Escherichia coli* O157:H7 spinach outbreak strain 4407 and *Salmonella enterica* serovar Newport) and examined the growth in the presence of the 6 broad spectrum fertilizers. The strains were grown overnight, centrifuged, and the pellets were resuspended in 3.0 ml of water. Aliquots of 300 µl (concentration approximately 5×10^8 cfu/ml) were added into 700 µl of each of the fertilizer solutions, and incubated for 24 h, 3 d, and 7 d. *E. coli* O157:H7 was plated onto Tryptic Soy agar (TSA) and Sorbitol-MacConkey agar (SMAC), and *S. Newport* was plated onto TSA and XLT4 agar. Positive and negative controls were plated at each time point. The colonies were counted after 24 h incubation at 35°C.

2.3.8 Fertilizers. The six fertilizers used in this study were calcium nitrate, ammonium sulfate, triple superphosphate, peters excel, ammonium nitrate, and urea. These fertilizers were mixed according to the manufacturer's directions in one liter of water: 5 g calcium nitrate, 4 g ammonium sulfate, 1 g triple superphosphate, 5 g peters

excel, 5 g urea, and 2 g ammonium nitrate.

2.3.9 Statistical Analysis. Each experiment was replicated three times using three samples for each treatment or dose plus three positive and two negative controls. Data obtained from individual experiments was considered independent. Significant differences will be determined using JMP statistical analysis software. The treatment effects on viability will be considered significant if $P \leq 0.05$.

2.4 Results

When HAV was suspended in liquid fertilizer, ammonium nitrate resulted in a nearly 2.5-log decrease in HAV after an incubation of 2 hours at 20°C. Triple superphosphate, peters excel, calcium nitrate, and ammonium sulfate resulted in a 1.5-log decrease in HAV at 20°C, and Ammonium Sulfate showed a 1.5-log decrease at 37°C (Figure 1). The remaining fertilizers resulted in ≤ 1 log reduction at the different time points and temperatures.

When HAV was attached to a solid surface, both triple superphosphate and calcium nitrate resulted in a 3-log reduction of HAV after an incubation of 3 days at 37°C (Table 3a). Urea and ammonium nitrate both resulted in almost 2.5-log reduction at this same time point. Ammonium sulfate and peters excel were shown to result in almost 2-log reduction of HAV after 3 days at 20°C and 37°C, respectively. Ammonium sulfate resulted in an almost 1.5-log reduction after 10 minutes of incubation at 20°C, and triple superphosphate and peters excel exhibited similar reductions after 7 days at 37°C. The remaining fertilizers exhibited reductions of ≤ 1 log at the remaining time points.

When AiV was suspended in fertilizer, peters excel exhibited a 2-log reduction after incubation of 3 days, and slightly short of 2-log reduction after an incubation of 2 hours (Table 4). At 2 and 24 hours, triple superphosphate exhibited 1.5-log reduction of AiV. The remaining fertilizers resulted in ≤ 1 log reduction of AiV at the specific time points.

When AiV was attached to a solid surface, peters excel exhibited 2.5- and 2-log reductions at 20°C after incubations of 24 hours and 2 hours, respectively. Triple superphosphate resulted in a 2-log reduction after 3 days at 37°C, and a 1.5-log reduction after 7 days at 20°C. The remaining fertilizers exhibited ≤ 1 log reduction at the remaining time points and temperatures.

When the viruses were attached to the surface of the lettuce and exposed to fertilizers, both peters excel and triple superphosphate resulted in more than 2.5-log reduction of AiV. The remaining treatments resulted in 1.5-log reduction or less for both AiV and HAV.

Although the bacteria were plated on selective media as well as general TSA, only the selective media data is presented. *E. coli* 4407 was found to be the most sensitive to calcium nitrate and ammonium nitrate, with no growth found after incubations of 24 hours, 3 days, or 7 days. On the other hand, the *E. coli* did not respond to peters excel or triple superphosphate. *Salmonella* Newport also did not respond to peters excel or ammonium sulfate after 3 days incubation, and exhibited slight growth in the presence of calcium nitrate at 24 hours and 3 days, ammonium

sulfate at 24 hours, triple superphosphate at 3 days and 7 days, ammonium nitrate at 24 hours and 3 days, and urea at all points.

2.5 Discussion

For the most part, the viruses were found to survive treatment with the fertilizers, although the viruses were slightly more susceptible when attached to a solid surface. Virus inactivation by 1-log could be useful if lower levels of contamination occur. This may be due to the extra stress associated with drying or due to the ability of chemicals to interact with the viral capsid this way. Overall, it appears that HAV is more resistant to inactivation suspended in fertilizer as opposed to being attached to a solid surface. Ammonium sulfate was the most effective at inactivating HAV at 10m at 20°C, while calcium nitrate and triple superphosphate were effective after 3 d at 37°C. While ammonium nitrate, ammonium sulfate, triple superphosphate, and urea seemed effective in inactivating HAV-contaminated fertilizer on lettuce, it must be taken into consideration that the lettuce sample was not scraped or agitated prior to removing the liquid sample. It is possible that the HAV attached to the surface of the lettuce sample and was not detected in the liquid fertilizer samples removed from the lettuce and liquid mixture.

When considering AiV, it must be taken into account that virus survival was higher in the presence of ammonium sulfate when attached to a solid surface after 7 d at both 20°C and 37°C. On the other hand, AiV was more sensitive to the effects of calcium nitrate, ammonium nitrate, and triple superphosphate after 7 d attached to the solid surface. Virus particles interacting with the lettuce sample were inactivated by

ammonium nitrate, calcium nitrate, peters excel, triple superphosphate, and urea; however, the virus may have just attached to the sample, and therefore not be detected in the liquid. When suspended in liquid fertilizer, AiV exhibited sensitivity to triple superphosphate and peters excel after 3 d.

Overall, ammonium nitrate, triple superphosphate, and urea were the most effective in reducing infections of both HAV and AiV in the lettuce samples submerged in fertilizers; however, none of the fertilizers were statistically effective in reducing both HAV and AiV in liquid suspensions or attached to a solid surface. Additionally, ammonium sulfate was problematic in that it would not stay in solution, even when mixed according to the manufacturer's instructions. Because of this, it is possible that the data for this fertilizer may be improperly represented.

It is believed that the fertilizers did not affect the viruses due to the chemical makeup of the viruses, capsid profile, and arrangement. Picornaviruses attach to cell surface receptors, resulting in a conformational change in the viral capsid proteins, which releases myristic acid. This release results in a pore in the cell membrane, in which RNA is injected. In order to inactivate these viruses, the receptors that normally bind to the host cell must bind with something else. Studies have shown that metal ions may inactivate some viruses by binding electron donor groups on proteins or nucleic acids (Thurman and Gerba 1988). In a study conducted by Samuni et al. (1984), it was suggested that copper may inactivate some viruses via a modified site-specific Fenton mechanism, which produces hydroxide radicals. These radicals are believed to affect the peptide backbone of the viruses. The

fertilizers used may not have been effective in inactivating the viruses in this study due to a lack of free materials to bind to the virion receptors.

Another study examined the effects of silver on virus particles, and found that some viruses were resistant to heavy metal inactivation (Cliver et al. 1971). Based on studies such as these, it is believed that the differing sensitivities of enteric viruses to metal ions may be due to the inherent stability of the virion particle's molecular structure (Abad et al. 1994). Picornaviruses are believed to have more stable molecular structures than other enteric viruses, which may account for the apparent ineffectiveness of fertilizers in the inactivation of HAV and AiV (Abad et al. 1994). In another study conducted by Yahya et al. (1989), *E. coli* and *Streptococcus faecalis* numbers were decreased following exposure to copper and silver ions mixed with chlorine; however, when viruses were added to the same mixture, inactivation did not occur.

It is believed that viruses generally resist inactivation via normal disinfection, which follows with the results of this study. In this study, the viruses were found to survive treatment with the fertilizers; however, the viruses were slightly more susceptible when attached to a solid surface. The results of this study suggest that the use of fertilizers may not inactivate viruses in contaminated water to a significant extent.

TABLES

Table 1. Available estimates for burden of foodborne illness attributed to virus contamination of food. This table summarizes population-based estimates for foodborne viral illness. Note that different approaches were taken in each of these studies; it is therefore not possible to make a direct comparison between the outputs of each study (CDC 2008).

| Country | Population size (approx.) | Viral infections (x10 ³) | Bacterial Infections (x10 ³) | Bacterial Intoxications (x10 ³) | Parasitic Infections (x10 ³) | Burden of viral illness |
|-------------|---------------------------|--------------------------------------|--|---|--|-------------------------|
| USA | 300 million | 9200 | 3715 | 460 | 357 | 1 in 33 |
| Australia | 20 million | 470 | 886 | 64 | 66 | 1 in 43 |
| Netherlands | 16 million | 90 | 283 | 114 | 25 | 1 in 178 |
| UK | 60 million | 77 | 659 | 221 | 4 | 1 in 780 |
| New Zealand | 4 million | 17 | 86 | 15 | N/A | N/A |
| Japan | 126 million | 13.5 | 12.7 | 1.8 | N/A | 1 in 933 |

Table 2. Metal content in biosolids, manures, and chemical fertilizers in parts per million (Spicer 2002).

| | Biosolids | Poultry Manure | Beef Cattle Manure | Phosphate Fertilizer | 40 CFR 503 Limits |
|------------|-----------|----------------|--------------------|----------------------|-------------------|
| Arsenic | 5 | 13 | N/A | 11.3 | 75 |
| Cadmium | 4.4 | 2.4 | N/A | 65 | 85 |
| Copper | 425 | 465 | 36 | 56.5 | 4300 |
| Lead | 76 | 46 | N/A | 12.2 | 840 |
| Molybdenum | 12 | 19 | 4.94 | N/A | 75 |
| Nickel | 33 | 16 | N/A | 27.5 | 420 |
| Zinc | 735 | 602 | 129 | 240 | 7500 |

Table 3a. Nitrogen, phosphorus, and potassium content of fertilizers used in this study, in percentages of total makeup.

| Fertilizer | Nitrogen | Phosphorus | Potassium |
|------------------------------|-----------------|-------------------|------------------|
| Ammonium sulfate | 21 | 0 | 0 |
| Ammonium nitrate | 34 | 0 | 0 |
| Calcium nitrate | 15 | 0 | 0 |
| Urea | 45 | 0 | 0 |
| Peters excel | 21 | 5 | 20 |
| Triple superphosphate | 0 | 42 | 0 |

Table 3b. Composition of fertilizers used in this study

| Fertilizer | Ingredients | Composition (%) |
|-----------------------|---------------------------|------------------------|
| Ammonium Nitrate | Ammonium Nitrate | 99-100 |
| Ammonium Sulfate | Ammonium Sulfate | 99-100 |
| Calcium Nitrate | Calcium Nitrate | 98-100 |
| Urea | Urea | 99-100 |
| Triple Superphosphate | Magnesium Oxide | 99-100 |
| | Calcium Phosphate | |
| | Phosphoric Acid | 0-1 |
| | Quartz | <1 |
| Peters Excel | Ammoniacal Nitrogen | 7.3 |
| | Nitrate Nitrogen | 12.6 |
| | Urea Nitrogen | 1.1 |
| | Phosphate | 5 |
| | Potash (K ₂ O) | 20 |
| | Boron | 0.0262 |
| | Copper (Water soluble) | 0.0262 |
| | Iron (Chelated) | 0.105 |
| | Manganese (Water soluble) | 0.0525 |
| | Molybdenum | 0.0105 |
| | Zinc (Water soluble) | 0.0525 |

Figures

Figure 1. Log reduction of HAV in liquid suspension at 20°C and 37°C at 10 minutes, 2 hours, 24 hours, and 3 days compared to a control of DI water.

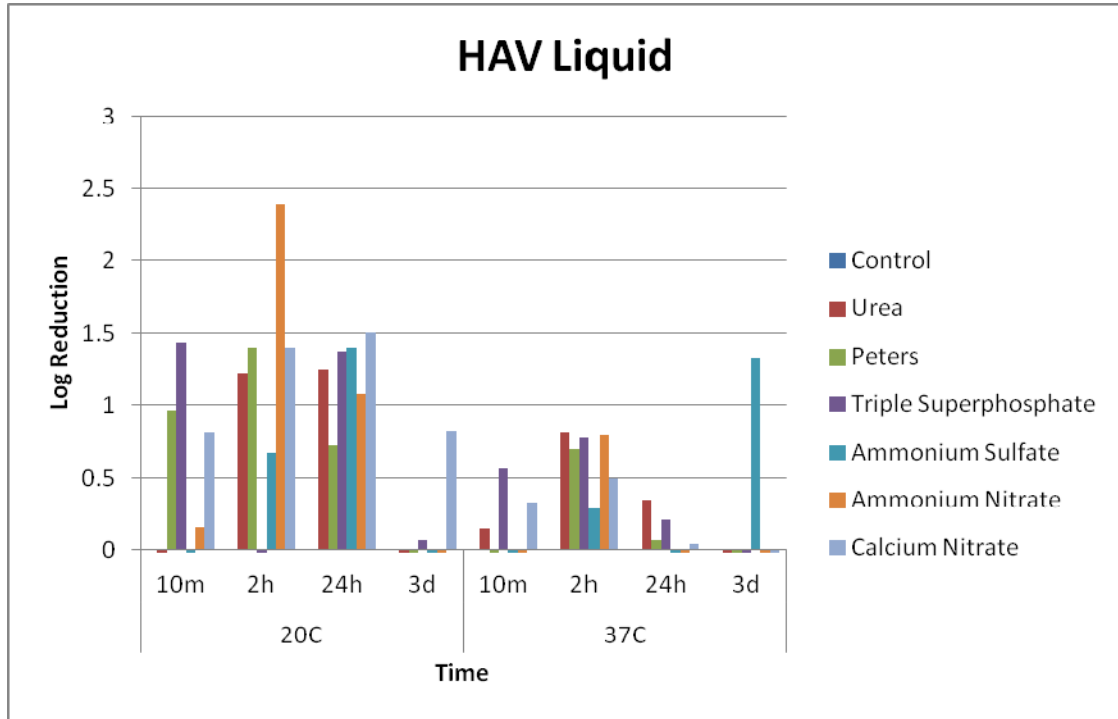


Figure 2. Log reduction of HAV attached to a solid surface at 20°C and 37°C at 10 minutes, 2 hours, 24 hours, 3 days, and 7 days compared to a control of DI water.

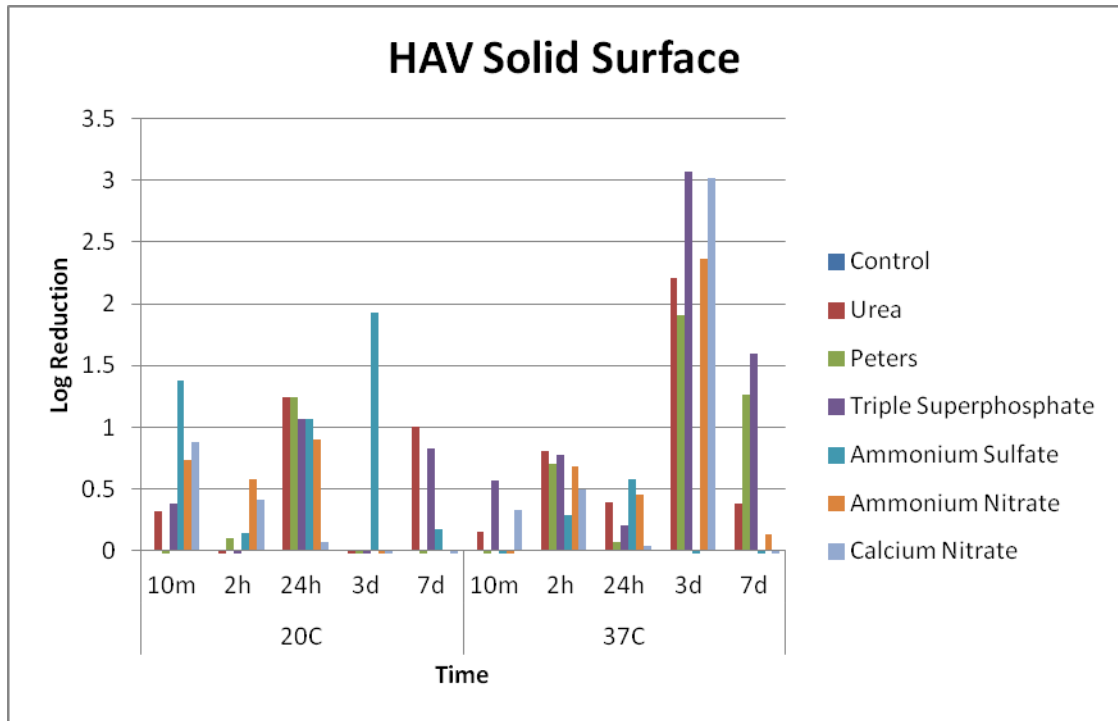


Figure 3. Log reduction of AiV suspended in liquid at 10 minutes, 2 hours, 24 hours, and 3 days at 20°C compared to a control of DI water.

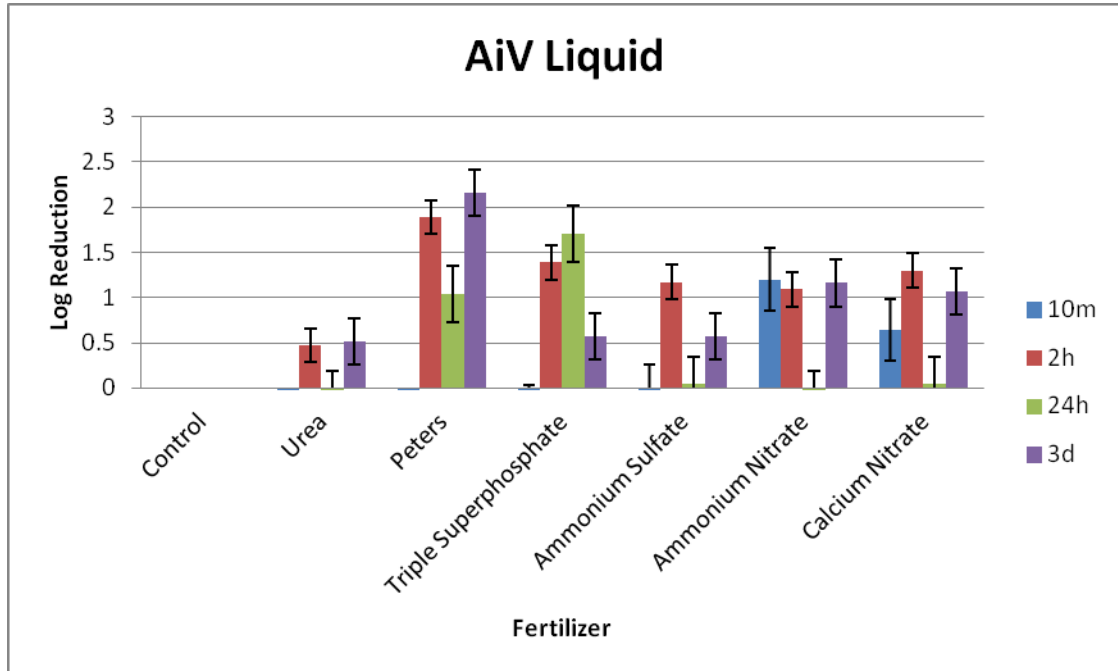


Figure 4. Log reduction of AiV attached to a solid surface at 10 minutes, 2 hours, 24 hours, 3 days, and 7 days at 20°C and 37°C compared to a control of DI water.

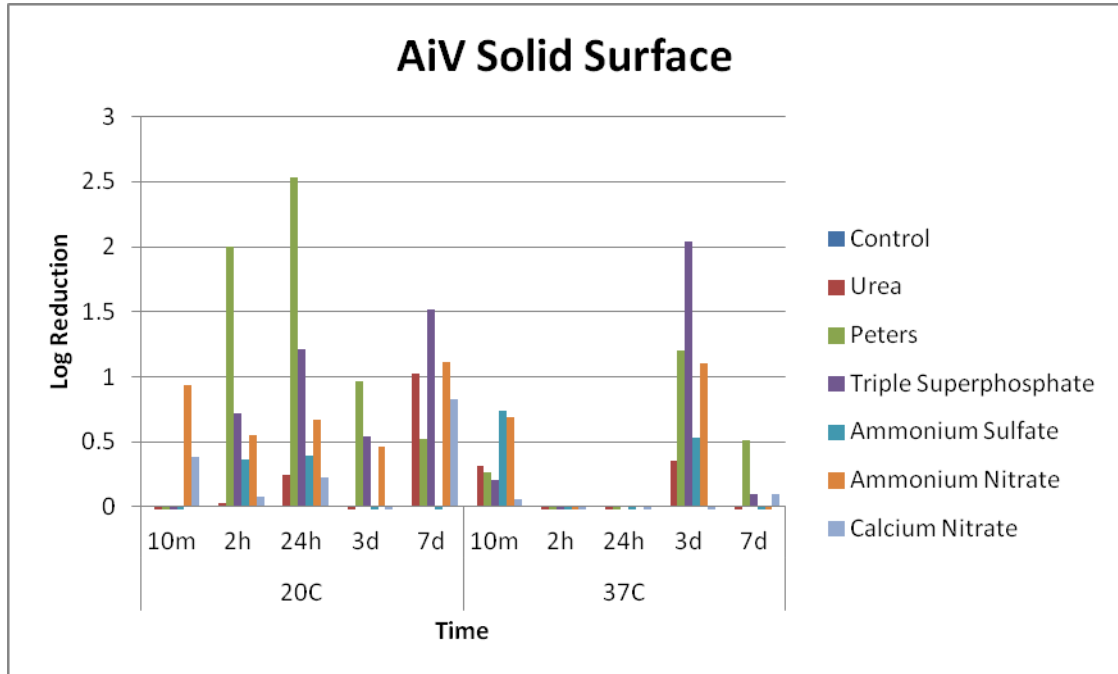


Figure 5. Log reduction of HAV and AiV after 3 days on lettuce at 4°C compared to a control of DI water.

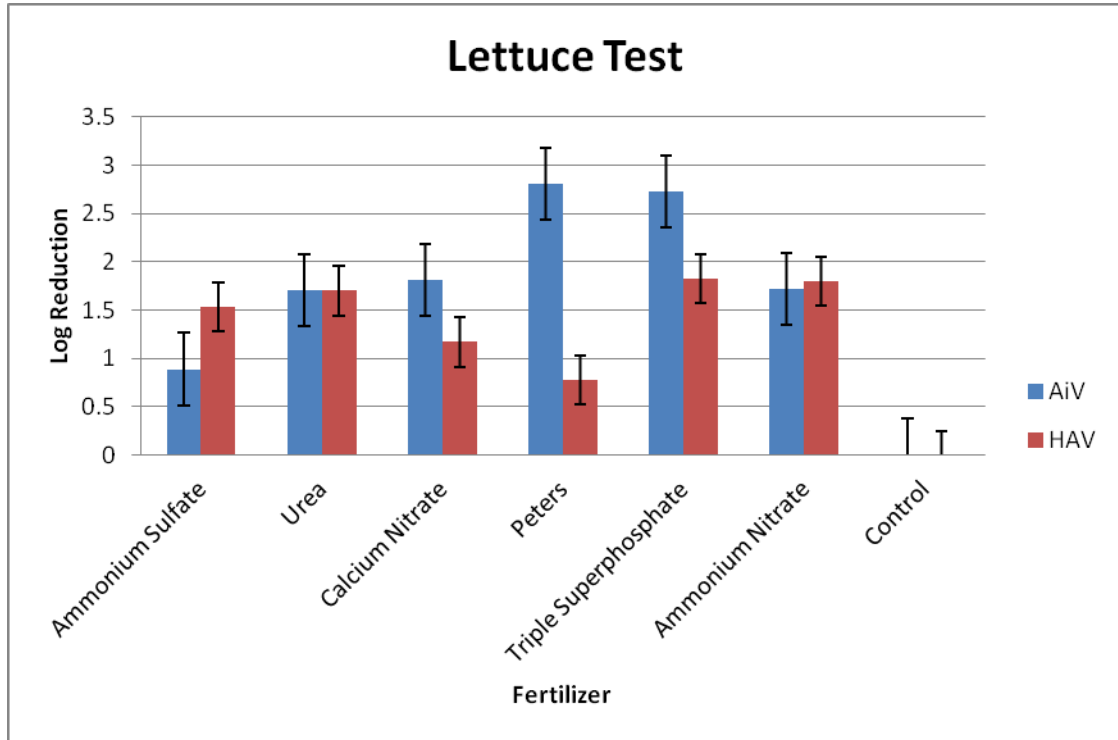
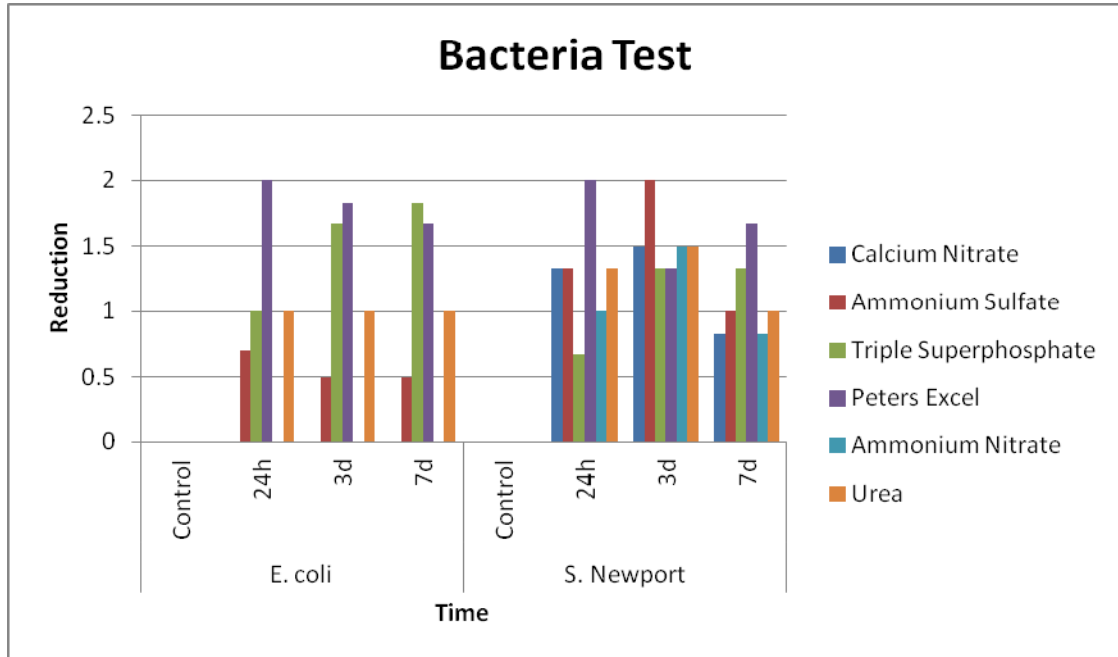


Figure 6. Reduction of *E. coli* 4407 and *S. enterica* serovar Newport at 24 hours, 3 days, and 7 days compared to a control of DI water.



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