ABSTRACT

Evaluation and Improvement of Methods to Recover and Detect Hepatitis A virus and an Indicator Virus (F+ RNA Coliphage MS2) in Representative Foods Associated with Viral Foodborne Outbreaks

Seventy-six million illnesses and 5,200 deaths are estimated to occur each year in the United States associated with foodborne transmission of microbial pathogens. Viral pathogens cause an estimated 7 out of 10 foodborne illnesses of known etiology. Improved methods to recover and detect viruses in foods may reduce morbidity and mortality associated with foodborne disease by identifying routes of viral transmission and by improving source tracking for foodborne outbreaks. Methods were developed and evaluated for recovery and detection of an enteric human virus, Hepatitis A virus (HAV), and an indicator virus, F+ RNA coliphage MS2 (MS2), in foods commonly associated with viral foodborne outbreaks. In this study, HAV was inoculated into tomato sauce, strawberries, and oysters, then recovered by either an acid-adsorption-elution-concentration (AA/E) method or an alkaline-elution-concentration method. Viruses were detected and enumerated by cell infectivity or by molecular methods. For molecular detection and quantification, viral RNA was extracted and amplified by conventional, nested, or realtime RT-PCR. The results of this study indicate that the AA/E method, initially used to recover low levels of enteric viruses from shellfish, may also be applied for the recovery of viruses from other complex foods, such as produce and sauces. Additionally, produce and sauces contain fewer enzymatic inhibitors to molecular detection than oysters. Because of this, larger produce and sauce samples can be analyzed by molecular detection methods, leading to possibly lower virus detection levels and greater assay sensitivity in these foods.

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50-TCID	50% tissue culture infectious dose
CDC	Centers for Disease Control and Prevention
CTAB	cetyltrimethyl ammonium bromide
BAM	Bacteriological Analytical Manual of the FDA
DAL	Double Agar Layer method
DEH	North Carolina Division of Environmental Health
DMF	North Carolina Division of Marine Fisheries
DNA	deoxyribonucleic acid
DWQ	North Carolina Division of Water Quality
EM	electron microscopy
FDA	Food and Drug Administration
FDC	Federal Food and Drug Cosmetic Act
GI	Norovirus genogroup I
GII	Norovirus genogroup II
GIII	Norovirus genogroup III
GIV	Norovirus genogroup IV
GuSCN	Guanidinium Isothiocinate
HAV	Hepatitis A virus
HIV	Human immunodeficiency virus
ID-50	dose to infect half of all patients
IEM	immune electron microscopy
MPN	most probable number
MS2	Coliphage MS2
NV	Norovirus
NSSP	National Shellfish Sanitation Program
PCR	polymerase chain reaction
PBS	phosphate buffered solution
PDU	polymerase chain reaction detectable units
PEG	polyethelene glycol
PVI	Poliovirus type 1

PFU	plaque forming unit
RNA	ribonucleic acids
RT	reverse transcription
RT-PCR	reverse transcriptase polymerase chain reaction
SAL	Single Agar Layer method
SRVS	small round structured viruses
TMDL	total maximum daily load
US	United States
USDA	U.S. Department of Agriculture

1 Introduction

Humans may be exposed to pathogens through many different routes including waste, water, air, soil, foods, vectors, or fomites. Among the estimated 38.6 million illnesses caused by known pathogens each year in the United States, an estimate 13.8 million illnesses (36%) result from foodborne transmission (Mead, Slutsker et al. 1999). It is also estimated that around 7 of every 10 foodborne illnesses of known etiology are caused by viral pathogens (Mead, Slutsker et al. 1999). Estimates for the number of viral foodborne outbreaks are used because; 1) only a fraction of foodborne outbreaks are reported and investigated (Olsen, MacKinnon et al. 2000); and 2) although technology exists for detecting non-cultivable viruses by polymerase chain reaction (PCR), the applied use of that technology to detect viral pathogens in foodborne outbreaks is seldom used— resulting in confusing labeling of viruses as "unknown agents" or "non-bacterial pathogens." This wrongful labeling de-emphasizes the importance of viruses in foodborne outbreaks, and leads regulators to make decisions without knowing all the facts. The data presented above by Mead and colleagues shows disease caused by known pathogens, but looking at the data from the point of view of unknown pathogens yield yearly estimates of 62 million (82%) illnesses and 3,400 (65%) deaths due to acute gastroenteritis caused by unknown foodborne agents (Mead, Slutsker et al. 1999). From this, one government employee comments that the possible economic costs of deaths due to

unknown agents is so large (around $17 billion per year) "that increased efforts to identify the causal agents are warranted" (Frenzen 2004).

Controlling foodborne outbreaks are important for other reasons than to maintain public health. There is an unknown, but high cost associated with most foodborne outbreaks. Patients made ill by foodborne outbreak face economic repercussions of their illness including loss of workdays, reduced quality of life, hospital visits, and in some cases death. Farms, distributors, restaurants and caterers also suffer economic losses from foodborne outbreaks traced to their operation. In fact, only 1 in 5 restaurants ever recover economically from suspected foodborne outbreaks (Jenkins 2004), so even "false alarms" have grave impact on restauranteurs and possibly others in the chain of food production and sale. Creating the causative link between foods and disease also applies to other fields such as food safety, bioterrorism, and forensic criminology. Such was the case with the purposeful *Salmonella* contamination of salad bars by a religious cult in Oregon (Torok, Tauxe et al. 1997).

Methods to recover and detect pathogenic viruses in the environment are complicated by low numbers of viruses that are dispersed non-randomly in matrices not easily amenable to virus recovery and detection. Given these challenges, science has made progress on enteric viral infection, replication, fate, transfer, and disinfection. In laboratory bench experiments, HAV and MS2, as single-stranded RNA viruses, may act as models for other single-stranded fecally associated viruses potentially contaminating foods. Research shows pathogenic viruses reach human hosts via the fecal oral route, and fecally contaminated foods are a significant mode of transmission providing viruses access to a host (Fankhauser, Noel et al. 1998). Once

contaminated foods are ingested, data suggests 1-1,000 virus particles are needed to cause an infection, using Norovirus as a model enteric virus (CDC-CaliciNet; Moe, Rhodes et al. 1998; Moe, Sobsey et al. 1999). With two common foodborne viral pathogens, infected individuals can develop symptoms of acute gastroenteritis in the case of Norovirus, and viral hepatitis from HAV. In symptomatic and possibly some asymptomatic infections, viruses are shed in levels as high as 10^9 particles of HAV per gram of feces and 10^8 particles of Norovirus per gram of feces (Koopmans and Duizer 2004); (Hollinger, Dienstag et al. 1991; Knipe and Howley 1991). Secondary and tertiary cases are common and often develop when an infected host spreads virus particles through environmental surface contamination and person-to-person transmission (Love, Jiang et al. 2002). In the event two or more unrelated individuals become ill from presumably the same source, epidemiologists label the event an outbreak.

During a foodborne outbreak, a local public health department may initiate an outbreak investigation (Jenkins 2004). In foodborne outbreak investigations, an epidemiologist will interview people involved in the outbreak to determine individual case histories, disease state, and foods consumed (CDC-Foodborne_Outbreak; Jenkins 2004). Depending upon available technology, viral pathogens, like Noroviruses, may be recovered and detected in a patients' stool using molecular methods (Jiang, Wang et al. 1992). Secondary and tertiary outbreak cases can then be linked to primary cases by matching the clinical case history, serological data, or genetic sequences of the virus recovered from patients. Epidemiologists also use clinical data and laboratory tests to identify the causative agent, but often only use statistical methods to identify contaminated foods. An important and weakly characterized link in the causative chain

of transmission is the microbiological content of the suspected food, which is rarely identified in the case of virally contaminated foods. This fact underscores why developing effective methods to recover and detect low concentrations of viral pathogens in food matrices is so important. With methods designed specifically to recover enteric viruses from foods, researchers will expose the etiology and taxonomy of many "unknown agents" in foodborne outbreaks.

1.1 General Objectives

The general objectives of this study were to develop and evaluate new and improved methods to recover and detect hepatitis A virus (HAV) and, for comparison, an indicator virus (F+ RNA coliphage MS2) from complex foods, including oysters, strawberry sauce and tomato sauce. The goal was to recover and concentrate these viruses by improved acid-adsorption elution methods. The recovered viruses were detected and quantified by molecular methods for nucleic acids using new techniques and commercially available kits for chemical extraction of ribonucleic acid (RNA). The goal was for HAV genomic RNA to be detected and quantified using nested and realtime RT-PCR for specific and sensitive detection at virus levels (with low detection limits).

1.2 Specific Objectives

The specific objectives of this study were as follows:

- To identify the best method for recovery of HAV and MS2 from oysters by elution in a comparison of 4 different candidate elution buffers.

- To determine and overcome the extent of food material inhibition of HAV detection in oysters, tomato sauce, strawberries processed by initial elution and acid-adsorption elution—when assayed by cell culture infectivity and realtime or conventional RT-PCR.

- To compare recovery of HAV from tomato sauce by acid-adsorption methods, with specific examination of three key method parameters: adsorption and elution pH levels, centrifugation conditions, and sample conductivity.

- To evaluate the use of a medium volume RNA extraction kit to concentrate and chemically extract HAV genomic RNA from larger sizes of food samples processed by acid-adsorption elution and elution methods.

- To validate and characterize the performance of standard procedures in semi-quantitative realtime RT-PCR detection of HAV RNA in foods.

- To successfully incorporate semi-quantitative realtime RT-PCR or nested RT-PCR as an improved method for sensitive detection and quantification of HAV RNA in food samples processed by the acid-adsorption elution method.

1.3 Experimental Design

This project aims to develop improved methods to recover, detect and quantify hepatitis A virus (HAV) and for comparison a fecal indicator virus, MS2, from foods commonly associated with foodborne viral gastroenteritis and viral hepatitis. The complex foods chosen for evaluation in this study were, oysters, tomato sauce, and strawberries, which have representative properties of many other complex food matrices, including sample consistency, pH, moisture content, fat, protein, and carbohydrate content.

The specific location of viral contamination in foods is the primary determinant of the recovery method employed. For example, surface contamination of pathogens on fruits or vegetables could be detected by eluting the pathogen from the skin or surface and then assaying the resulting eluate. Homogenizing a surface-contaminated food would not be advised for simple and efficient virus recovery. This would unnecessarily increase the sample size, reduce the sample quality, and effectively hide the pathogen within a mass of blended food tissue. However, internally contaminated foods, such as processed foods, salad bar foods, oysters, or processed fruits and vegetables cannot be assayed by surface elution techniques. Therefore, internally contaminated foods must be homogenized, or otherwise pulverized to expose the contaminated parts and their associated viruses. After the viruses are exposed within the food matrix, the virus can then be eluted, concentrated, purified, and detected.

In the present study, 25 to 30 grams of internally contaminated food samples were employed. These relatively small sample amounts were used as proof of concept for methods development, but were typical food sample portion ingested by an individual. Although, in foodborne viral outbreak investigation, it may be preferable to assay replicate 50 to100 gram samples, because viral contamination is usually at low concentrations and may not be randomly distributed with a batch or lot of food. In bench laboratory experiments, food samples were seeded with virus (HAV and/or MS2) and homogenized to distribute the virus evenly and to liquefy the sample. Food samples were processed so as not to degrade or rupture the virus capsid until the RNA extraction step was to be applied to a purified and concentrated sample.

Efforts to recover viruses from homogenized foods focus on two commonly employed types of methods: 1) elution of the virus using an alkaline buffer; and 2) acid-adsorption of the virus onto foods, followed by elution using an alkaline buffer. In each method, a stepwise analytical approach was employed to determine the efficiency of each step of elution, concentration, and the influence of sample quality throughout the steps of the procedure.

Within investigation of the elution step, experiments were designed to assess 4 eluents for their ability to recovery viruses (HAV and MS2) from oysters, tomato sauce, and strawberries. The eluent media and method best suited for recovering virus was identified for further experiments. Next, the effect of sample quality of the food eluate on virus detection was investigated by making serial tenfold dilutions of the sample in water or phosphate buffered solution (PBS) and then assaying for viruses by cell culture and reverse transcription-

polymerase chain reaction (RT-PCR). The final sample quality must be compatible with the method of virus detection. Efforts were made to achieve adequate quality of the sample at intermediate steps in sample processing quality in order to check for the efficiency of virus recovery at each critical processing step in the method. After resolving questions on the effects of alternative processing on sample quality, the detection limit of the virus (HAV and MS2) in the processed food sample was determined using the developed elution method.

An acid-adsorption elution method for virus recovery, concentration and purification was investigated in a similar manner as for the elution method. The virus recovery and detection efficiency of steps in the acid adsorption method was characterized, and alternative experimental methods were then designed to improve upon poor virus recovery or small sample sizes of existing methods. Specifically, the ability of viruses to be efficiently adsorbed to tomato sauce was studied in depth. Variables investigated for their effects on virus adsorption and elution included pH and conductivity of samples, the geometry of the centrifugation tube (conical bottom or round bottom), and centrifugation force used to separate the sample and recover viruses in one of the resulting separated phases (sedimented solids or overlying supernatant liquid). Virus concentration from the resulting supernatant using polyethylene glycol (PEG) precipitation was investigated and conditions of this processing step were optimized for virus recovery. Subsequent extraction of HAV RNA from the resuspended PEG precipitate were investigated for the ability to process large food sample volumes using silica-based spin columns.. Efforts were made to determine the effects of sample quality on virus recovery and detection by realtime RT-PCR. Detection and quantification of viral RNA by this method was compared to that of nested RT-PCR. The entire acid-adsorption method developed by these investigations was then be used to determine

the lower detection limit of HAV recovery and detection in tomato sauce and strawberry samples. Efforts to fully characterize the performance of these methods for HAV detection in large sample sizes of oysters were also investigated.

2 Literature Review

2.1 Introduction to the Literature Review

The literature review is arranged in four major sections. Section 2.2 describes enteric viruses and fecal indicator bacteriophages important in foodborne outbreaks and food safety research. Two important foodborne enteric viruses, HAV and Norovirus, and one fecal indicator virus, F+ coliphage MS2 are described in detail. Section 2.3 presents a general review of foodborne outbreaks, and describes laboratory methods for recovering enteric viruses and fecal indicator viruses from contaminated foods. Section 2.4 is an in depth review of shellfish, highlighting how shellfish can act as a model for food safety research and regulations. Section 2.5 is a summary of the literature review.

2.2 Viruses

2.2.1 Introduction

Viruses are obligate intracellular parasites that infect a host cell for purposes of replication and in some cases reprieve. Viruses contain a nucleic acid core; surrounded by a protein capsid, and in some cases a lipid envelope. Viral genetic material may be in the form of deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) in single or double stranded configurations. This hereditary information is encapsulated in a symmetrical or variable

pattern of capsomeres arranged to form the capsid. Viruses may be transmitted from host to host, reservoir to host, or from the environment to a host. Viral infections produce a wide range of effects, from asymptomatic infections to local or chronic morbidity, and even mortality. Host mediated antibody defense serves as active protection from viral infection in some organisms. Of interest to foodborne safety are fecally associated viruses that are common, harmful, and easily transmissible to humans. Two such enteric foodborne viruses, HAV and Norovirus, are reviewed below. The structural morphology, clinical symptoms, infectious dose, risk groups, and documented outbreaks are explained for each virus. Bacteriophages, or viruses infecting bacteria, are also described with a detailed section on Coliphage MS2, a fecal indicator virus.

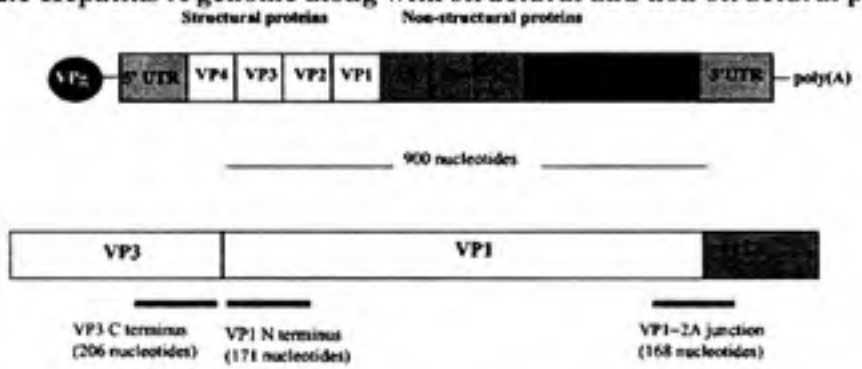
2.2.2 Hepatitis A Virus

2.2.2.1 Hepatitis A Virus Structure

Hepatitis A (HAV) is a virus in the *Hepatovirus* genus, contained within the *Picornaviridae* family of non-enveloped, positive sense single stranded RNA viruses. HAV was first classified as *Enterovirus* type 72, but due to differences in nucleotide sequence, poor growth in cell culture, and resistance to conditions that would inactivate other picornaviruses, some authors would wish for the reclassification of HAV as a separate genus (Knipe and Howley 1991). Other species within the *Picornaviridae* family are included in the genera *Rhinovirus* and *Enterovirus*. HAV was first visualized, and thus formally identified in 1973 by Feinstone and colleagues (Feinstone, Kapikian et al. 1973). The hepatitis virion is 27-32 nm in diameter and has a nucleotide sequence 7.5 kb long. The HAV genome has a single open reading frame

encoding for the structural proteins VP1, VP2, VP3, and VP4 and the non-structural proteins, such as RNA dependent RNA polymerase, associated with virus synthesis and replication (Figure 2.2.1) (Costa-Mattioli et al., 2003). There are eleven well-characterized strains of HAV collected from primates and humans, with one, HM175 (Australia, 1976) used in this study (Knipe and Howley 1991).

Figure 2.2.1 Hepatitis A genome along with structural and non-structural proteins.



Picture taken from (Costa-Mattioli et al., 2003).

2.2.2.2 Environmental Stability

HAV is stable at pH 1.0 for 2 hours at room temperature, and will retains its infectivity for up to 5 hours. Other Picornaviruses do not fair so well, and lose infectivity after 2 hours at pH 1.0. HAV is resistant to some level and duration of heat. The virus is resistant at 60°C for 1 hour, partially inactivated at 10-12 hours ((Perry and Mortimer 1984; Siegl, Weitz et al. 1984), and inactivated in minutes at 98-100°C (Provost, Wolanski et al. 1975). In longer term trials, HAV retains infectivity when dried and stored at 25°C and 42% humidity for 1 month (McCaustland, Bond et al. 1982; Sobsey, Shields et al. 1988), and may remain infective indefinitely when stored at -20°C (Knipe and Howley 1991). Natural environs where HAV may survive for months or longer are in artificially contaminated fresh water, seawater,

wastewater, soils, marine sediments, live oysters, and crème-filled cookies (Sobsey, Shields et al. 1988). HAV is inactivated by a variety of chemicals or mechanisms including ultraviolet radiation, autoclaving, formalin, iodine, or chlorine, with specific thresholds for duration and intensity (Knipe and Howley 1991). Inactivation by chlorine is of particular interest because water treatment plants often use chlorine to reduce the pathogen load in drinking water. In a review article it was noted HAV requires 10-15 ppm of residual chlorine over 30 min, free residual chlorine at 2.0-2.5 mg/L for 15 minutes (Coulepis, Anderson et al. 1987).

2.2.2.3 Clinical

Viral hepatitis, caused by HAV, contains four distinct clinical phases: 1) incubation; 2) preicteric stage; 3) icteric phase; and 4)convalescent period (Knipe and Howley 1991). Viral hepatitis has a median incubation of roughly one month, but ranges from 15-50 days (Chin, CCD Manual). Incubation period may decrease with a high dose of virus (Paul, Havens et al. 1945). Infection lasts 1-2 weeks in mild cases, and in severe cases is disabling for months (Chin 2000). Symptoms of viral hepatitis include anorexia, nausea, malaise, fever, abdominal discomfort, and headaches. Patients may also develop asymptomatic cases, termed inapparent or sub-clinical hepatitis, producing neither symptoms nor jaundice (Knipe and Howley 1991). Although two-thirds of clinical cases happen in children and young adults, around 70% of deaths from the disease occur in individuals >50 yrs (Knipe and Howley 1991).

2.2.2.4 Transmission

Hepatitis A is a common cause of viral hepatitis, accounting for nearly two-thirds of all viral hepatitis cases (CDC 1999a). The CDC estimates 31% of people in the US have ever been infected with HAV (CDC 1999b). In the US, an estimated 270,000 HAV infections have occurred annually from 1980 to 1999, which is 10.4 times greater the number of cases than was actually reported (Armstrong and Bell 2002). Rates of viral hepatitis are higher in developing countries with crowded living conditions and poor sanitation, with 100% of children acquiring immunity at an early age (Knipe and Howley 1991). Although HAV is endemic in much of the developing world, as sanitation and hygiene improve, HAV infection is delayed (Knipe and Howley 1991). In the US, a high proportion of the population is susceptible to HAV infection.

The most important mode of transmission of HAV is though person-to-person contact (CDC 1999b). In one model of HAV transmission, of every 100 cases of viral hepatitis in 6-11 year olds, 47 separate secondary infections would occur within households (Meyerhoff and Jacobs 2001) from person-to-person contact. HAV is spread predominantly through the fecal-oral route, and can be considered an enterically transmitted disease (Knipe and Howley 1991). From 2 to 7 weeks post-exposure and before clinical symptoms begin; the virus is present in the stool of infected individuals—at most 10^9 virions per gram feces. (Hollinger, Dienstag et al. 1991; Knipe and Howley 1991). Shedding of HAV in stool may last for months after clinical symptoms have halted (Yotsuyanagi, Koike et al. 1996).

Foodborne transmission is also well documented. Foodborne infections have arisen from field-contaminated produce (Rosenblum, Mirkin et al. 1990; Calder, Simmons et al. 2003), virus uptake by shellfish (Dienstag, Gust et al. 1976; Desenclose, Klontz et al. 1991; Xu, Li et al. 1992; Anonymous 1997; Bosch, Sanchez et al. 2001), and from infected foodhandlers contaminating items like salad (Hooper, Juels et al. 1977), bread (Warburton, Wreghitt et al. 1991) meats and cheeses (Gustafson, Hutcheson et al. 1983; Parkin, Marzinsky et al. 1983). In one study, lettuce was experimentally contaminated by a volunteer's finger pad. Of a 1.29 X 10^5 plaque forming units (PFU) of HAV finger pad inoculum, 7% or roughly 9000 PFU was transferred and recovered from lettuce samples (Bidawid, Farber et al. 2000a). Virus transfer was reduced 30-fold with the use of topical agents or alcohol between finger pad contamination and lettuce inoculation (Bidawid, Farber et al. 2000a).

Though present, HAV transmission through blood is rare, as is waterborne transmission, exposure to urine, nasopharyngeal secretions, and by biting insects (yet unconfirmed) (Knipe and Howley 1991; Cuthbert 2001).

2.2.2.5 Infectious dose

Early studies on the infectious dose of HAV were carried out in the middle of the last century during human dosing studies in prisoner populations, and mentally disabled children's institutions. Since then, human dosing trials of HAV have been deemed unethical and the only information available to estimate infections dose is from animal models and some circumstantial outbreak information. .

In animal models, routes of exposure and infectivity of HAV were investigated in 2 species of primates, showing wild-type HAV was $10^{4.5}$-fold less infectious by the oral route compared to intravenous dosing, thus $10^{4.5}$-fold more virus was needed to infect by the oral route (Purcell, Wong et al. 2002). These results highlight an intestinal barrier to infection from HAV in primates, and possibly humans (Purcell, Wong et al. 2002). Similarly, in experimentally infected chimpanzees, the onset and duration of viremia was related to quantity of innoculum (Bower, Nainan et al. 2000). These data relate the hypothesized dose-dependent relationship for HAV infections in human and animal models. Using circumstantial outbreak information an inverse relationship was found between the numbers of sandwiches consumed and the incubation period for ill individuals during an outbreak of HAV at a picnic caused by contaminated tuna sandwiches (Istre and Hopkins 1985).

Other work attempts to infect non-human primates with human stool samples. Polish and colleagues qualified the amount of HAV present in human stool by ranking samples as positive or negative with various molecular detection tools (1999) (Table 2.2.1). Samples scored as highly, moderately and weakly reactive were dosed to Tamarins, a species of primate, with varying responses (Table 2.2.1). Only highly reactive human stool samples produced infections in test animals (Polish, Robertson et al. 1999).

Table 2.2.1 Relation between HAV positivity from human stool and infection in animals.

Scale of HAV Reactivity	Detection signal in human stool		Infectious response in Tamarins (# 1-6) dosed with human stool					
	EIA	PCR-ETBr	1	2	3	4	5	6
Highly reactive	+	Strongly +	+	+				
Moderately reactive	-	Weakly +			-	-	-	-
Weakly reactive	-	-						

+ = positive for detection or infection
- = negative for detection or infection
Table compiled from data in (Polish, Robertson et al. 1999)

2.2.2.6 At risk groups

More than half of patients infected with hepatitis A can be categorized into one or more groups with recognizable risk factors (CDC 1996). At risk groups given as a percent of all reported infections in a 1993 study show that 22% of cases are from personal contact with HAV infected patient; 5% from homosexual activity; 16% from child/employee at day-care or contact with one; 6% from International travel; 2% from food or waterborne routes 2% from IV drug use (CDC 1996). In a review article on HAV risk factors, Franco and colleagues recommend the following groups be vaccinated: "blood transfusion recipients, travelers, the military, healthcare workers, sewage workers, foodhandlers, day care assistants, institutionalised subjects, blood transfusion recipients, drug addicts, homosexuals, prisoners and other risk groups such a liver transplantees" (Franco, Giambi et al. 2003).

2.2.2.7 Hepatitis A outbreaks associated with foods

HAV has been linked to viral hepatitis outbreaks involving many types of foods such as: shellfish, finfish, produce, baked goods, meats, dairy products, and mixed foods (Table 2.2.2). Contamination of foods with HAV may occur at any level from 1) growing; 2) harvesting; 3) processing, preparation, and value added-production. Environmental contamination of HAV in shellfish, and specifically filter-feeding bivalve mollusks is well documented (Dienstag, Gust et al. 1976; Desenclose, Klontz et al. 1991; Xu, Li et al. 1992; Anonymous 1997; Bosch, Sanchez et al. 2001). Bivalve mollusks may accumulate viruses in higher concentrations than in ambient growing waters, due to both an advanced system for capturing particles in suspension, including virus associated particles, (Table 2.2.2) (Hedstrom and Lyke 1964; Hoff, Jakubowski et al. 1965; Metcalf and Stiles 1965; Mitchell, Presnell et al. 1966), and an ability to filter hundreds to thousands of gallons weekly (CBF; VA-DEQ; Sobsey and Jaykus 1991). A startling outbreak of viral hepatitis in foods was due to HAV contamination in shellfish around Shanghai, China, sickening 300,000 (Xu, Li et al. 1992). Produce eaten fresh such as blueberries or lettuce or frozen fruits like strawberries and raspberries, and foods lightly cooked like green onions have been implicated in HAV outbreaks with contamination often originating at the farm level (Rosenblum, Mirkin et al. 1990; Calder, Simmons et al. 2003). Other outbreaks involving fresh produce result from foodhandler contamination during food preparation (Hooper, Juels et al. 1977; Latham and Schable 1982). Prepared foods, such as hot-dogs, bread, potato salad, sandwich foods, and other mixed foods contaminated by foodhandlers have also been implicated in HAV outbreaks (Osterholm, Kantor et al. 1980; Gustafson, Hutcheson et al. 1983; Hayashi, Yagi et al. 1988; Lowry, Levine et al. 1989; Warburton,Wreghitt,et.al.1991).

Table 2.2.2 Foodborne transmission of Hepatitis A virus by food type.

Reference	Location	Source	Cases
		Shellfish	
Bosch 2001	Valencia, Spain	clams from Peru	183
Anonymous 1997b	NSW, Australia	Oysters	500
Xu 1992	Shanghai, China	raw clams	300,000
Desenclose 1991	5 states, USA	raw oysters	61
Dienstag 1976	Victoria, Australia	incompletely cooked mussels	10
		Finfish and Prawns	
Anonymous 1997a	Sydney, Australia	frozen fresh-water prawns at restaurant, but not linked to foodhandler	23
Istre 1985	–	tuna salad at a picnic	11
		Produce	
MMWR, 2003	Several states, USA	green onions	207
Calder 2003	New Zealand	raw blueberries by pickers	39
Dentinger 2001	Ohio, USA	green onions from farm	43
Hutin 1999	Michigan and Maine, USA	frozen strawberries	242
Niu 1992	multi-state, USA	frozen strawberries	28
Rosenblum 1990	Kentucky, USA	lettuce, contaminated before distribution	–
Reid 1987	Aberdeen, UK	frozen raspberries	24
Latham 1982	Utah, USA	green salad from foodhandler	23
Hooper 1977	California, USA	tossed salad & fresh grapefruit from foodhandler	133
		Baked Goods	
Becker 1996	Euskirchen, Germany	baked goods	49
Warburton 1991	Cambridge, UK	bread contaminated by foodhandler	>50
		Meats and Dairy	
Gustafson 1983	Tennessee, USA	cold meats and cheeses from foodhandler	small #
		Multiple foods	
CDC 2003	Massachusetts, USA	Uncooked & cold food from foodhandler	43
Lowry 1989	Florida, USA	green salad, pantry food, mixed drinks from restaurant	103
Hayashi 1988	Nagoya, Japan	lunches from a supplier	–
Osterholm 1980	--	ice, potato salad, molded salmon, & hotdogs from foodhandler	102

Modified from Graham et al., 2000; Jaykus 1997; and PubMed search

Although common matrices for foodborne viral hepatitis outbreaks are understood, the most likely sources of foodborne contamination (farm-based, environmental, foodhandler based) are not well characterized. Environmental contamination of shellfish caused the largest known HAV outbreak, but farm-based and foodhandler-based transmissions have caused single outbreaks involving hundreds of cases (Hooper, Juels et al. 1977; Osterholm, Kantor et al. 1980; Hayashi, Yagi et al. 1988; Xu, Li et al. 1992; Hutin, Pool et al. 1999). Resolving the risk of source-based association of foodborne contamination is of critical importance for the remediation of foodborne outbreaks. One reason the scientific community has difficulty addressing this question is that published peer-reviewed articles blur the line between causal modes of pathogen contamination and transmission. To resolve this, viral outbreak investigations should include common methods. To these ends, the Centers for Disease Control and Prevention (CDC) has issued a web-based "Outbreak Investigation Toolkit" providing protocols for sample collection, case identification, a patient questionnaire, and a form for state or local health departments to report foodborne outbreaks (CDC-Foodborne_Outbreak). Unfortunately, the CDC model lacks advice on how to collect and process foods implicated in viral outbreaks, as do many state and local health departments (CDC 2003) (Love, SS personal communication).

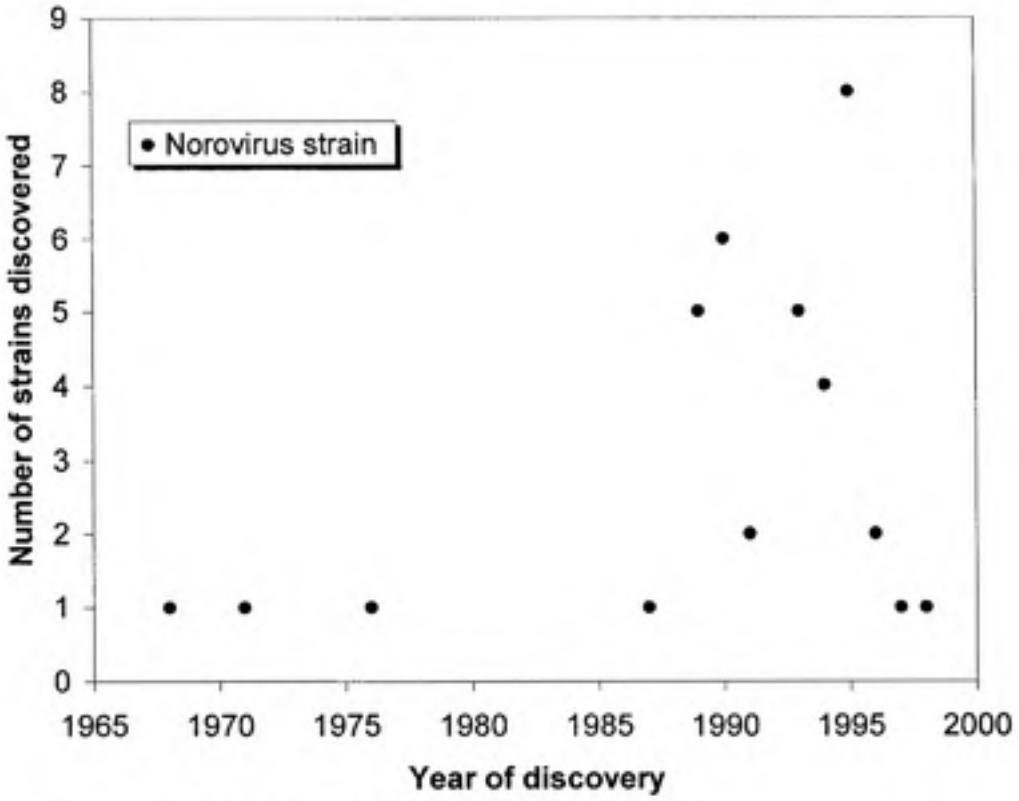
2.2.3 Norovirus

2.2.3.1 Norovirus Structure

Noroviruses are a nonenveloped, icosahedral virus 27-40 micrometers (μm) in size. As a human Calicivirus, it is within the family *Caliciviridae*. Within the capsid of Noroviruses resides a linear, positive sense, single stranded RNA genome. Norwalk virus, the first of many Noroviruses discovered, was first visualized by electron microscopy (EM) in 1972 by Kapikian and colleagues (1972). This virus strain was collected 3 years earlier in an outbreak at a school in Norwalk, Ohio (Adler and Zickl 1969)— thus the virus's namesake. EM was employed in early efforts to characterize Norwalk virus and other morphologically recognizable viruses. It was also in this era Rotavirus was visualized using EM in duodenal mucousa from challenged patients (Bishop, Davidson et al. 1973; Bishop, Davidson et al. 1974).

Noroviruses are separated into four groups, genogroups I, II, III, and IV (GI, GII, GIII, and GIV) based on phylogenic analysis, with GII the most common in norovirus outbreaks. Figure 2.2.2 shows the number of Norovirus strains discovered each year, over a twenty-year period. The peak in the number of new norovirus strains discovered in the1990s is due to advances in molecular methods including genome cloning of Norwalk virus, sequencing of a norovirus, detection by reverse transcriptase polymerase chain reaction (RT-PCR) and development of genogroup specific primers (Jiang, Wang et al. 1992; Ando, Monroe et al. 1995).

Figure 2.2.2 Norovirus strains discovered over 20 years, 1969-1998.



Data from Fields Virology (Knipe and Howley 1991).

2.2.3.2 Clinical

Norovirus associated viral gastroenteritis may assail all ages of humans. The disease presents itself with acute-onset vomiting in most cases. Other symptoms include watery non-bloody diarrhea, abdominal cramps, nausea, and in one-third to one-half of all patients, a low-grade fever for < 24 hours. Further symptoms are myalgia, headaches, chills, and general malaise. Incubation takes 1-2 days, and symptoms generally persist for 1-3 days, but may be prolonged in elderly patients. A common complication of Norovirus associated gastroenteritis is dehydration, especially in elderly and young patients. Patients generally make a complete recovery with no sequelae. Asymptomatic infections occur in up to 30% of infections. In non-specific cases (non-outbreak or

unrecognized outbreak cases) Norovirus associated viral gastroenteritis is difficult to identify on clinical grounds only. Diagnosis of the disease is aided greatly by an epidemiological investigation that take into account timeliness, location, transmission type, virus strain, and other suspected links between cases.

2.2.3.3 Transmission

Norovirus is transmitted from person-to-person, foodborne, waterborne, from the environment, and from fomites. In many instances, outbreaks may involve multiple pathways (Love, Jiang et al. 2002). The virus infects at low doses, has a high infectious rate, infected individuals may shed high amounts of virus in stool (Höhne and Schreier 2004). Norovirus has a high infection rate, with 82% becoming infected in a clinical dosing trial involving 50 people (Graham, Jiang et al. 1994). Persons infected with norovirus may shed large quantities of the virus in stool, as high as 10^8 particles per gram of feces reported in a review article (Koopmans and Duizer 2004). Virus shedding usually occurs with the onset of symptoms (CDC-CaliciNet) and can extend for up to 2 weeks (Bresee, Widdowson et al. 2002). It has also been shown that shedding begins before symptoms present themselves. In one study, a food handler free of symptoms infected 48 restaurant patrons, yet did not become ill himself until after his shift (Gaulin, Frigon et al. 1999). The issue of shedding is also important for asymptomatic carriers of the virus.

2.2.3.4 Infectious dose

Scientific logicians assume 1 virus particle as a single entity and the minimum quantity required for infecting humans, but the exact number of Norovirus particles required to infect humans in a dose-response study is much higher and ranges within 2 logs. Before the early 1990s and the advent of RT-PCR detection for Norovirus (Jiang, Wang et al. 1992; Jiang, Wang et al. 1992), estimates of infectious dose of Norovirus were artificially inflated due to poor sensitivity of detection. Current estimates predict as few as 10 virus particles are needed for infection (CDC-CaliciNet), but other studies cite a higher infectious dose. Using human volunteers, Moe and colleagues report 6000 virus particles (Moe, Rhodes et al. 1998) and 100 virus particles (Moe, Sobsey et al. 1999) are needed for infection— but both reports appeared in non-peer reviewed forums. Two Norovirus review articles ascribe to Moe and colleagues' 1999 findings (Glass, Noel et al. 2000; Bresee, Widdowson et al. 2002), and possibly strengthening the case for such results. Further work by Moe (2001) gave the dose to infect half of all patients (ID-50) as 430 PCR detectable units (PDU) (Moe 2001). As technology improves, ID-50 and/or infectious dose in patients may steadily decline. With present technology one may assume that infectious dose may be as low as 1 and 1,000 virus particles.

2.2.3.5 At risk groups

Norovirus may infect all ages, but is of specific concern to certain risk groups. Risk groups are subsets of a population with a higher than average likelihood of incurring disease. Risk groups may be associated with a given activity, lifestyle, age, or any other variable. One method for characterizing risk in Norovirus related gastroenteritis is

through epidemiological investigations. In a broad study by Fankhauser and colleagues, 90 outbreaks of non-bacterial gastroenteritis over 18 months were investigated using RT-PCR with two primer sets, GI and GII (Ando, Monroe et al. 1995). Ninety-six percent of outbreaks were caused by Norovirus, and consumption of contaminated food was identified most (37%) as the mode of transmission (Fankhauser, Noel et al. 1998). Activities or locations that were implicated in a high percentage of outbreaks are listed in Table 2.2.3. One must also note the size of the outbreak and the number of persons at risk, before labeling at risk groups. While a greater percentage of outbreaks occur in nursing homes and hospitals (43%) than at other settings, which on average sickened 42 people and put 147 people at risk, less common "vacation setting" outbreaks (11%) affect an order of magnitude more people (Fankhauser, Noel et al. 1998). An interpretation of the results in Table 2.2.3 is that any Norovirus outbreak sufficiently robust as to occur in multiple locations within similar sub-sets of a population should be considered a risk.

Table 2.2.3 Ninety non-bacterial gastroenteritis outbreaks from 1996 to 1997.

Location	Total Outbreak Percentage of outbreaks
Nursing homes and hospital	43%
Restaurant and catered meals	26%
School and day care centers	11%
Vacation setting including cruise ships	11%
Oyster consumption	6%
Other (prison and homeless shelter)	3%
Total	100%

modified from Fankhauser et al. 1998

2.2.3.6 Norovirus outbreaks associated with foods

Norovirus outbreaks are in many ways similar to HAV outbreaks, in that both are non-enveloped single stranded RNA viruses of similar size, are transmitted by the fecal-oral route, and infect at very low copy numbers. Norovirus has a much shorter incubation period (1-2 days) than HAV (15-60 days), and is shed in stool as early as 15 hours post-infection with a peak shedding at 25-72 hours (Graham, Jiang et al. 1994). Rapid infection and subsequent viral replication enables a faster turnaround time for re-infection or transmission to another host. Although initial norovirus outbreaks often involve foodborne or waterborne transmission, infected individuals then spread the virus quickly to others by person-to-person contact or person-environment-person contact.

Norovirus outbreaks may occur in any matrix. Listed in Tables 2.2.4 and 2.2.5 are recent viral gastroenteritis outbreaks from various matrices with cases confirmed to be norovirus positive. Transmission is listed as foodborne, waterborne, or the location where the outbreak occurred, such as hotels or restaurants. Table 2.2.4 lists the magnitude of outbreaks as the number of cases. Importantly, facilities that hold large numbers of individuals are also susceptible to large outbreaks. Such is the case in both 378 and 587 person outbreaks on cruise ships (McEvoy, Blake et al. 1996; Thornton, Davies et al. 2002), a 300 person outbreak at a concert hall (Evans, Meldrum et al. 2002), a 116 person outbreak at a hotel (Love, Jiang et al. 2002), a 112 person outbreak at a restaurant (Mertens, Thijs et al. 2002), and both 281 and 450 person outbreaks at nursing homes (Miller, Carter et al. 2002; Fretz, Schmid et al. 2003). To address the likelihood of a norovirus related gastroenteritis outbreak in various sources, CDC analyzed 348

outbreaks during 1996-2000, and found more occurred through foodborne transmission (38%) than by any other means (Fankhauser, Noel et al. 1998). Person-person contact was identified in 12% of norovirus gastroenteritis outbreaks, and waterborne transmission in 3% of outbreaks (Fankhauser, Noel et al. 1998). Within the same data set, outbreaks occurred most often in nursing homes and hospitals (43%), followed by restaurants or catered meals (26%) (Fankhauser, Noel et al. 1998). This information provides an insight into the risk of a viral gastroenteritis outbreak occurring among institutional and non-institutional health, foodservice, and recreational settings. Further work to characterize and document norovirus outbreaks is ongoing at CDC through passive surveillance in both the Foodborne Outbreak Response and Surveillance unit and CaliciNet, a database of norovirus sequences used to link outbreaks (CDC-CaliciNet; CDC-Foodborne_Outbreak).

Table 2.2.4 Norovirus Transmission in Recent Viral Gastroenteritis Outbreaks.

Reference	Location	Transmission and/or Source	Number of outbreaks	Cases
		Foodborne		
Furuta 2003	Hamamatsu City, Japan	Clams	1	22
Berg 2000	multiple states, USA	Oysters	3	418
Shieh 2000	California, USA	Oysters	1	171
Le Guyader 1996	7 states, USA	Oysters	25 clusters in 7 states	>130
Kohn 1995	Na	Oysters	1	70
Daniels 2000	Texas, USA	deli sandwiches	1 restaurant	23
Girish 2002	Delhi, India	Salad sandwiches	1	130
Griffin 1982	New Jersey, USA	Salad	2	63
Holtby 2001	UK	Salad	1	6
Gaulin 1999	Quebec, Canada	Raspberries	1	>200
Almagro, Nievas 2003	Granada, Spain	Whipped cream	1	218
Gotz 2002	Sweden	pumpkin salad	30 day care centers	195
Lopman 2003	England and Whales	Fish	6	Na
Lopman 2003	England and Whales	Meat	5	Na
Lopman 2003	England and Whales	Oysters	20	Na
Lopman 2003	England and Whales	Poultry	9	Na
Lopman 2003	England and Whales	salads and vegetables	17	Na
Lopman 2003	England and Whales	Other *	29	Na
		Waterborne		
Marks 2003	Na	Water vapor	1 school	Na
Boccia 2002	Southern Italy	Drinking water	1 resort	344
Nygard 2004	Norway	Drinking water	13	Na
Anderson 2003	Wyoming, USA	well water	2 lodges	35
Kukkula 1999	Heinavesi, Finland	Municipal water	Town	estimated 1700-3000
Schwoerer 1999	France	Tap water	1 hospital	2 or more
		Hotels		
Love 2002	Virginia, USA	food; environment; person-to-person	1	116
		Restaurants		
Mertens 2002	Netherlands	person-to-person	2 groups	215
Peipins 2002 **	Virginia, USA	Food; person-to-person	Multiple	45

Na = not available, * "other foods" includes fruit, soup, desserts, and savory snacks, ** samples from a restaurant and buildings

A search on PubMed for "Norovirus Outbreaks" returned 452 hits. This table represents some findings from this search.

Table 2.2.5 provides data on a special type of environmental transmission of norovirus, from sewage and/or human feces polluting shellfish growing waters. Shellfish may accumulate viruses in greater concentrations than ambient waters, and shellfish contaminated with enteric viruses may cause viral gastroenteritis, such as the thousands of norovirus cases in Table 2.2.5. Themes from Table 2.2.5 will be discussed in more detail in the section on shellfish.

Table 2.2.5 Oysters related outbreaks due to Norovirus and (pre-harvest) sewage contamination.

Reference	Location	Date	Source of Sewage	Cases
Appleton, 1977	England	1976	Unknown	33
Murphy, 1997	Australia	1978	Unknown	2000
Gill, 1983	England	1983	Unknown	137
Kohn, 1995	Louisiana, USA	1993	oyster harvester	190
CDC MMWR 1994	Florida, USA	1993	oyster harvester	30
CDC MMWR 1995	Florida, USA	1995	oyster harvester, recreational boaters	70
Berg 1997	Louisiana, USA	1996	Oyster harvester, oil rig	72
CDC, MMWR 1997	Louisiana, USA	1996	oyster harvester	153

Modified from (Berg, 2000)

2.2.4 Bacteriophages

2.2.4.1 Introduction

Bacteriophages (phages) are viruses that infect bacteria, as opposed to other types of viruses that infect animals or plants. Phages were first discovered in the early 20th century by British and French researchers (Pelczar, Chan et al. 1988). Phages have similar structural and morphological characteristics to other viruses, namely, a protein coat surrounding a nucleic acid core. Phages may contain single stranded or double stranded RNA or DNA (Table 2.2.6). Phages selectively infect hosts depending upon the receptor sites on the surface of bacteria (Goyal, Gerba et al. 1987). With phages that

infect bacterial host *Escherichia coli*, called Coliphages, classification of family is based on the methods of viral attachment. Somatic coliphages attach to host cell walls, while male-specific (F^+) coliphages attach to host sex pilli (Hsu, Shieh et al. 2002). Once conformation between phages and host, viral replication occurs only inside metabolizing host bacteria, through the use of host ribosomes, amino acids, protein synthesizing factors, and energy (Goyal, Gerba et al. 1987).

2.2.4.2 Bacteriophages as Indicators

In early 20th Century researchers attempted unsuccessfully to use bacteriophages (phages) as antibacterial agents to treat bacterial diseases (Goyal, Gerba et al. 1987). The main reason bacteriophages are still studied today is the morphological and structural similarities between phages and enteric viruses. Phages, and more specifically coliphages, serve as a fecal indicator virus in aquatic environments (Hsu, Shieh et al. 2002). Criteria for an ideal indicator of fecal contamination are 1) an applicability to sample matrix; 2) present in feces, sewage, and fecally contaminated samples; 3) correlate the amount of pathogens in fecally contaminated samples; 4) has no regrowth in the environment; 5) survives and persists greater than or equal to pathogens; 6) is easily and quickly detected and quantified by short lab tests; 7) is harmless to humans and animals; and 8) the number of indicators relate to a risk of enteric illness (Bonde 1963; Bonde 1966; Sobsey 2003). Although coliphages do not fulfill all requirements of an ideal fecal indicator, they do agree in points 1, 2, 5, 6, 7, and including point 4 for male-specific (F^+) only. A list of important families of phages that may be used as fecal indicators is listed in Table 2.2.6.

Table 2.2.6 Bacteriophages important for water quality assessment.

Family	Nucleic Acids	Typical Members	Tail
Leviviridae	ssRNA	enterobacteriophage MS2, Qß	no
Inoviridae	ssDNA	Coliphage f1, fd, M13	no
Microviridae	ssDNA	Coliphage fX174	no
Podoviridae	dsDNA	Coliphage T6	yes
Siphoviridae	dsDNA	Coliphage T5	yes
Myoviridae	dsDNA	Coliphage T4	yes

modified from (Grabow 2001)
data from (Maniloff and Ackermann 1988; Pelczar, Chan et al. 1988)

Environmental monitoring studies have investigated phages listed in Table 2.2.6 as fecal indicators in various matrices such as shellfish (Dore and Lees 1995; Chung, Jaykus et al. 1998; Dore, Henshilwood et al. 2000; Formiga-Cruz, Allard et al. 2003), ground beef and poultry (Hsu, Shieh et al. 2002), and water (Havelaar, van Olphen et al. 1993; Chung, Jaykus et al. 1998; Skraber, Gassilloud et al. 2004). Dore and colleagues working with shellfish and shellfish growing waters found a positive association between high levels of F^+ RNA bacteriophage contamination and both fecal pollution in growing waters and shellfish-associated disease outbreak (2000). Similarly, others have found that the presence of enteric viruses was correlated with the phage *Bacteroides fragilis* strain RYC2056 and male specific F^+ RNA phages in contaminated shellfish samples (Formiga-Cruz, Allard et al. 2003; Muniain-Mujika, Calvo et al. 2003). Importantly, in shellfish monitoring studies, some samples were positive for enteric viruses but contained no indicator phage (Formiga-Cruz, Allard et al. 2003). In some cases the lack of correlation between phages and enteric viruses may be due to the methods used to analyze respective organisms. Using molecular methods, enteric virus particles are detectable in assays sometimes tens to hundreds of times more sensitive than standard plaque-based detection methods for phages. As Grabow summarizes, the presence of phages merely indicates

the potential for a similar presence of enteric viruses; and the absence of phages merely indicates the potential for a similar absence of enteric viruses (2001).

2.2.4.3 Coliphage MS2

Coliphage MS2 is an icosahedral positive-sense single stranded F^+ RNA coliphage in the Leviviridae family (Grabow 2001). The complete genome of MS2 is ~3.5 kb in length and is available using a BLAST search engine. MS2 has similar diameter, structure, and nuclear properties to human enteric viruses like hepatitis A virus, norovirus, and enterovirus. Resistance of MS2 to chemicals, heat, sunlight, ultra-violet light, chlorination, and water treatment processes is well documented, as reviewed by Grabow (2001). The use of MS2 as a viral indicator of enteric viruses in matrices such as water and foods is still unclear and may take years to decades to amass enough environmental monitoring data to validate. But, MS2 can be used in bench experiments to easily, quickly, and cheaply act as a model or surrogate for enteric viruses. MS2 is enumerated visually using three methods: 1) a single-agar-layer (SAL) plaque assay, where molten agar is combined with the sample and log-phase host bacteria, then poured onto a petri dish (EPA method 1602) (Sobsey, Schwab et al. 1990; Sobsey, Battigelli et al. 1995); 2) a double-agar layer (DAL) method similar to SAL but molten agar mixture is poured onto solidified agar (EPA method 1602)(Sobsey, Schwab et al. 1990; Sobsey, Battigelli et al. 1995); 3) a spot-titer culture method, where bacteriophage is spotted in 10ul amounts on a solidified agar and host plate (Josephs, Coulleitte et al. 2004), 4) most probable number (MPN) enrichment, 5) and molecular methods (Vinje et al., 2004). MS2 is also detected

using molecular techniques of RT-PCR and probe hybridization (Hsu, Shieh et al. 1995; Oudejans, Long et al. 2003).

2.3 Foodborne Outbreaks and Virus Detection

2.3.1 General review of foodborne outbreaks

Among the estimated 38.6 million illnesses caused by known pathogens each year in the United States, ~36 % or 13.8 million of those resulted from foodborne transmission (Mead, Slutsker et al. 1999). Foodborne contamination may occur at any point from the farm to the fork (Koopmans, von Bonsdorff et al. 2002), and to predict the exact point at which contamination occurs is difficult. Foodborne transmission of pathogens may involve large outbreaks; the largest documented outbreak sickened 300,000 in Shanghai, China with hepatitis and was caused by contaminated shellfish (Xu, Li et al. 1992). Other important settings for foodborne outbreaks are hospitals, schools, commercial settings, and gatherings with lax foodhandler or food storage regulations like picnics, potlucks, or parties. Foodborne illnesses vary by pathogen type, dose, and host susceptibility. Nevertheless, it may be assumed that any episode of duress caused by a foodborne pathogen is undesirable, and could compromise future health, wage-earning capability, and quality of life of the primary host or any other downstream case.

Many foodborne illnesses are underreported, with reported cases showing the tip of the iceberg. Surveillance studies from Centers for Disease Control and Prevention (CDC) from 1983-1992 lists around 5,000 foodborne outbreaks infecting around 170,000 patients (Bean, Griffin et al. 1990; Bean, Goulding et al. 1996). In the patients where an

etiological agent was identified, bacterial pathogens comprised 90-92% and viral pathogens only 5-6% (Bean, Griffin et al. 1990; Bean, Goulding et al. 1996). In the last decade, presumably the nature of foodborne outbreaks have not have changed significantly from those recorded by CDC in the 1980s and early 1990s, but recently the number of foodborne viral illnesses has increased. In recent years, laboratories have acquired the ability to detect genomic information from previously classified "non-bacteria samples" by polymerase chain reaction (PCR). Previously unidentified or difficult to identify foodborne pathogens may now be categorized as viral pathogens. It is estimated that currently around 7 of every 10 foodborne illnesses are caused by viral pathogens (Mead, Slutsker et al. 1999). The application of molecular biology has revolutionized the strain-specific identification and sequence matching of cases of gastroenteritis and other foodborne illnesses.

Tracking foodborne outbreaks and illnesses provides useful information for healthcare professionals, regulators, commercial food distributors, and the general consumer public. FoodNet, an epidemiological and environmental monitoring system for common pathogens associated with foodborne outbreaks was spearheaded in 1995 by CDC, the US Department of Agriculture (USDA), and the Food and Drug Administration (FDA). Foodnet is an important first attempt at tracking foodborne cases of bacterial and parasitic organisms in the United States (US) population, but lacks a nationwide monitoring of even the most common foodborne viral pathogens. As current technology provides opportunities for improvements in food safety, manageable systems regulating the detection, tracking, and reduction of pre-consumer foodborne pathogen contamination is

an achievable goal. Towards these ends a corporate entity, a government, and a society has the knowledge, technology, and responsibility to secure safe and healthful foodstuffs.

2.3.2 Methods for detecting viruses in foods

Testing foods for viral pathogens is not a routine operation, and often performed only with just cause, for example, when epidemiological evidence links the consumption of foodstuffs with an outbreak or if the source of infection is desired (Cliver, Ellender et al. 1983a). Even among known outbreaks, identifying the mode and source of contamination is difficult, because foods may become contaminated anywhere "from farm to fork" (Koopmans and Duizer 2004). Food-stuffs may come in contact with viruses from human feces during irrigation, harvesting (Rosenblum, Mirkin et al. 1990; Calder, Simmons et al. 2003), washing (Warner, Carr et al. 1991), or food preparation (Hooper, Juels et al. 1977; Latham and Schable 1982; Gustafson, Hutcheson et al. 1983; Warner, Carr et al. 1991; CDC 2003), while other foods like shellfish actively accumulate viral pathogens by filtering fecally polluted seawater (Sheih, Baric et al. 2003). Foods as vehicles for pathogens pose varying risks depending upon several factors such as pathogen-host characteristics, dose, and degree of cooking. The type of pathogen contaminating the food and an individuals response to the pathogen dictates the severity of response. For example, Botulinum toxin (from *Clostridium botulinum*) in baby food (Armada, Love et al. 2003) can prove fatal for infants, while a gastrointestinal pathogen may only cause mild diarrhea and dehydration. The amount of contaminated food consumed may increase the severity or duration of disease as shown in an outbreak of HAV at a picnic, where an inverse relationship was drawn between number of

contaminated sandwiches consumed and the incubation period of the illness (Istre, 1985). Processing or cooking regimes will also modify risk of disease by decreasing pathogens in food as reviewed by Koopmans and colleague (2004), or by introducing a time for foodhandlers to contaminate food (Warburton, Wreghitt et al. 1991; CDC 2003) for example). Methods to detect pathogens in foods aid in determining viral loads in contaminated foods, outbreaks or otherwise; and by relation, help public health professionals and regulators attenuate patterns of food-borne contamination and consumption among high-risk populations.

Detecting viral pathogens in foods is challenging, time consuming, and expensive (Cliver, Ellender et al. 1983a). Detection limits may vary with food-type, pathogen, amount of contamination, or detection method as shown by Casteel (2001) and reviewed by Jaykus for shellfish (Jaykus 1997; Casteel 2001). Methods for detecting viruses may be inhibited by entities in the food— especially with molecular detection. Samples producing inhibitors to molecular detection include, but are not limited to soil-covered produce (Casteel 2001), internally contaminated foods and/or complex foods that requires homogenization (Casteel, Love et al. 2003), certain plant polysaccharides (Demke and Adams 1992), and shellfish (Atmar, Metcalf et al. 1993). Inhibitors to reverse transcription RT or PCR may give false negative signals in molecular detection. In addition, many viruses common to foodborne outbreaks do not grow or grow poorly in cell culture (Cromeans, Sobsey et al. 1987; Atmar and Estes 2001), and few animal models are available (Polish, Robertson et al. 1999; Purcell, Wong et al. 2002).

Types of foods commonly linked to viral gastroenteritis or viral hepatitis outbreaks are shellfish (Desenclose, Klontz et al. 1991; Anonymous 1997; Berg, Kohn et al. 2000; Furuta, Akiyama et al. 2003), produce (Gaulin, Frigon et al. 1999; Hutin, Pool et al. 1999; Dentinger, Bower et al. 2001; Calder, Simmons et al. 2003) baked goods (Warburton, Wreghitt et al. 1991; Becker, Promse et al. 1996), deli sandwiches (Daniels, Bergmire-Sweat et al. 2000) and mixed salads (Holtby, Tebbutt et al. 2001; Gotz, J.B. et al. 2002). With imprecise epidemiological data and challenging environmental confirmation, multiple foods can be implicated in a single outbreak. Such was the case for 1) Lowry and colleagues with the potential culprit being green salad, pantry food, or mixed drinks from restaurant (Lowry, Levine et al. 1989); 2) Osterholm and colleagues with ice, potato salad, molded salmon, and hotdogs implicated in an outbreak (Osterholm, Kantor et al. 1980); and 3) restaurant food among others to blame for an outbreak exposed by Love and colleagues (Love, Jiang et al. 2002). Lack of correlation between illness and a single food type in the examples above may mean multiple sources of contamination, but most likely not enough epidemiological evidence was gathered to pinpoint the cause of the foodborne illness. In these examples and in other outbreak investigations, environmental detection may be useful to secure the etiological link between a likely contaminated food-type and morbidity.

Suspected contaminated foods from outbreaks are collected according to routine sampling procedures outlined in the Bacteriological Analytical Manual (BAM) by FDA's Center for Food Safety and Applied Nutrition (FDA 2003) and by the International Commission on Microbiological Specifications for Foods (ICMSF 1986). Briefly, BAM

asks for a statistically significant number of representative food samples (at least 100g each) from the purportedly contaminated food to assure meaningful results (FDA 2003). Sampling varies by food-type, with solid, semisolid, viscous, or liquid foods (FDA 2003). Sterile sampling technique and aseptic equipment must be used to collect the food samples (FDA 2003). Food samples may be kept in the original packaging or stored in "clean, dry, leak-proof, wide-mouthed, sterile, and of a size suitable for samples of the product" container (FDA 2003). Food samples are then transported to a laboratory in the original storage conditions (frozen, refrigerated, or at room temperature) and analyzed within 24 or 36 hrs for shellfish and refrigerated foods respectively (FDA 2003). Once arriving at the laboratory for processing, various methods are employed to detect and/or recover pathogens from food samples. A detailed and practical review of such methods for viral pathogens will ensue.

2.3.2.1 Recovery, extraction and concentration

Processing regimes for detecting viral pathogens in food samples depend heavily on the type of contamination and the type of food. Samples may have surface contamination, such is often the case with whole fruits, nuts, and vegetables (Cliver, Ellender et al. 1983a; Rosenblum, Mirkin et al. 1990; Warner, Carr et al. 1991; Holtby, Tebbutt et al. 2001; Calder, Simmons et al. 2003), or internally contaminated as with shellfish or mixed foods (Reid and Robinson 1987; Atmar, Neill et al. 1995). Foods contaminated by a foodhandler may contain either surface or internal contamination, depending on the food type and amount of food preparation. Once the type of contamination is determined, an effective recovery scheme may be employed.

Briefly, with surface contamination on produce items like lettuce and berries, viruses may be eluted with simple surface washes (Konowalchuk and Spiers 1977; Bidawid, Farber et al. 2000a; Casteel 2001; Dubois, Agier et al. 2002). The produce-washed eluates generally contain some of the following list of reagents: salts, buffer components, acids or bases, and proteins as summarized by Cliver and colleagues (1983a). In early work, grapes were surface-contaminated with poliovirus 1 and coxsackievirus B5, then virus was eluted comparably with water, phosphate-buffered saline (PBS), or 0.5 % polyethylene glycol (Konowalchuk and Spiers 1977). Later studies by Bidawid and colleages also used PBS, in this case 1 ml of slightly basic PBS (pH 7.6) was pipetted/washed 25 times on produce inoculated with HAV (Bidawid, Farber et al. 2000b). After concentration, investigators could detect HAV by RT-PCR when as little as 10 PFU was inoculated initially on lettuce. In another surface-elution approach, intact produce samples were gently agitated (100 rmp) in 30 ml of buffer for 60 minutes to release surface contaminated virus particles (Casteel, 2001). Using larger food samples than other researchers, Duboins and Agiers recovered PV, HAV, and NV from 100 g of raspberries, fruit mixture, strawberries, radishes, and lettuce (Dubois, Agier et al. 2002). Fruit and produce were relatively free of inhibitors to RT-PCR in undiluted samples, but some inhibition was seen. Anywhere from 0.2-0.06 g of fruit and produce was assayed in one RT-PCR reaction, which corresponds to ~0.2 % of the total food sample. The detection limit by RT-PCR was an input level of 50 TCID (50% tissue culture infectious dose) of HAV when inoculated in 100 grams of food. The method was able to recovery 17% of initially inoculated HAV. Regardless of the simplicity or bench-based

proficiency, few surface-elution methods have been used to detect virus particles in outbreak situations. Schwab and colleagues used a novel and successful approach to detect NV from outbreak samples by RT-PCR (2000). To recover noroviruses and HAV, they washed 30 g of contaminated deli meats (ham, turkey, and roast beef) with either 40 ml of PBS or soaked in 4 ml of TRIzol (a guanidinium-phenol-based reagent) (Schwab, Neill et al. 2000). PBS washed samples were fluorocarbon extracted and PEG concentrated, as done with typical elution methods, while in the faster TRIzol approach no virus concentration was necessary. The TRIzol method was chosen, giving deli meat samples were free of inhibitors to RT-PCR at the 1:100 dilution and only 0.06 g (0.2%) of food was assayed per RT-PCR reaction. The detection limit by RT-PCR was reached at an input level of 0.4-4 PFU of HAV in 30 grams of deli meat, and HAV was always detected by RT-PCR with input levels of 5-50 PFU. One fault of this new technique is that processed samples are only applicable to detection by molecular methods and not by cell-culture infectivity, because virus capsids are lysed early in the method. This deficiency precludes the Schwab method from use in disinfection experiments, or to recover enteric viruses that have animal models for detection.

Internally contaminated foods require more rigorous processing than surface contaminated foods. Typical processing of internally contaminated foods begins with blending, stirring, or shaking the sample in liquid. Foods of varying texture, solidity, or make-up may require more or less homogenization to achieve an adequate mixture. In general, homogenization increases the surface area of the food and allows an eluate more contact with viruses. As Cliver and colleagues note, the homogenate should not be

unduly heated during mixing (1983a). Following this advice, previous studies have homogenized food in chilled, sterile water (Chung, Jaykus et al. 1996; Shieh, Calci et al. 1999; Mullendore, Sobsey et al. 2001). After homogenization, a researcher has several options among two popular types of methods and many lesser known methods to process foods.

2.3.2.2 Elution-Concentration

Two popular methods, most notably used for virus detection in shellfish, are elution-concentration and adsorption-elution-concentration (also known as acid-adsorption-elution-concentration). The former method employs pH changes towards basic on homogenized sample, thus enabling the elution of virus particles from the food matrix. Samples are typically centrifuged and the virus containing supernatant is recovered, then further clarified and concentrated before cell-culture assay or nucleic acid detection. For an in-depth review of extraction-concentration from its inception in the mid-1960s (Jaykus, Hemard et al. 1994). One criticism of the extraction-concentration method is the lack of clean supernatant samples, which may inhibit virus detection (Jaykus, Hemard et al. 1994).

Responding to valid concerns, researchers attempt overcome poor sample quality by modifying the way foods were processed. In one study, 50 grams samples of lettuce and hamburger were inoculated with poliovirus type 1 (PV1), HAV, and Norovirus (NV) and recovered by homogenization-elution-concentration (Leggitt and Jaykus 2000). Hamburger extracts were filtered through cheesecloth after elution to remove impurities,

while "cleaner" foods like lettuce received no filtration (Leggitt and Jaykus 2000); (Sair, D'Souza et al. 2002). On average, 2-4 % HAV was recovered using this method. Interestingly, hamburger samples were free of inhibitors to RT-PCR at the 1:10 dilution (~ 0.15g of hamburgers per reaction), while lettuce samples required an extra tenfold dilution (~ 0.015 g lettuce per reaction). Surprisingly, the more inhibitory material to RT-PCR had a lower detection limit (1,000 PFU HAV in initial 50g lettuce) than that for hamburger (10,000 PFU in initial 50g hamburgers). This example highlights the differences between recovery and detection; namely, a method may be advanced in means to recover viruses (as in the case for lettuce), but may be deficient in means to detect virus particles by RT-PCR (so too with lettuce). Sample quality, as the compatibility of a sample with a method for detection, and in this case RT-PCR by Leggitt, is often achieved through extensive dilution of final samples. Another way to improve sample quality is to start with smaller samples, as reached by dissecting oysters stomachs, digestive diverticula, and/or hepatopancreas instead of processing whole oyster (Atmar, Neill et al. 1995; Atmar, Neill et al. 1996). Dissected oysters provide a 1.5g single sample size (Atmar, Neill et al. 1995), which are purported to contain fewer inhibitors than whole oyster samples (Shieh, personal communication). Another finding supporting the use of dissected oysters is that viruses are localized in oyster digestive systems—as described in Section 2.4.7. Regardless of the choice to dissect shellfish, for clean samples researchers must also pursue aggressive clarification steps such as one or more fluorocarbon extractions and one or more concentration steps using 5% cetyltrimethyl ammonium bromide (CTAB), Cat-Floc T, Pro-Cipate, or polyethelene

glycol (PEG) (Atmar, Metcalf et al. 1993; Jaykus, De Leon et al. 1996; Mullendore, Sobsey et al. 2001).

Samples processed as above hopefully contain high concentrations of viruses in a small volume of liquid buffer, with virus recovery and inhibition removal dictated by the steps of a method. Small volume final samples (0.1- 1 ml) are favored for detection by molecular methods, while larger final volumes (1-10 ml) are also acceptable in infectivity assays.

2.3.2.3 Acid-Adsorption Elution-Concentration

A second popular method to concentrate virus particles found in foods, but mainly shellfish, is adsorption-elution-concentration. The method originated in 1978 (Sobsey, Carrick et al. 1978), and a quarter of a century later a version of this method (Mullendore, Sobsey et al. 2001) was found to be most sensitive when compared to six other methods (Atmar, Neill et al. 1996; Chung, Jaykus et al. 1996; Hafliger, Gilgen et al. 1997; Green, Henshilwood et al. 1998; Lees 2000; Kingsley and Richards 2001) in a monitoring study in Switzerland (Beuret, Baumgartner et al. 2003). In another comparative study, Suñén and colleagues also found acid adsorption elution preferable over an elution method for recovering HAV from clams (Sunen, Casas et al. 2004).The acid-adsorption elution method, in its most current revision, has a detection limit of 0.33 PFU of HAV seeded in oysters per RT-PCR reaction (Mullendore, Sobsey et al. 2001). This method typically uses whole oysters (*C. virgincia*), which weigh ~10-25g and are processed in 25g or 50g batches (Chung, Jaykus et al. 1996; Jaykus, De Leon et al. 1996; Shieh, Calci et al. 1999; Mullendore, Sobsey et al. 2001). The method has also been performed effectively with

clams (Sunen, Casas et al. 2004). Briefly, homogenized shellfish are adjusted to pH ~4.8-5 and a conductivity <2,000 μS/cm, centrifuged, and the acid-adsorbed virus pellet is retained (Mullendore, Sobsey et al. 2001). Optimization of the conductivity and pH of the shellfish homogenate for recovery of enteric viruses was undertaken in the early to mid 1970s (Sobsey, Wallis et al. 1975). Subsequent single or multiple elutions using a slightly basic (pH 7.5-9.5), saline (0.14 M NaCl), protein solution (e.g. 0.05 M glycine or 0.5 M threonine) elute virus off oyster material. Large volume eluates are concentrated with single or multiple PEG precipitations (4-10% PEG, usually 8% and 0.3 M NaCl) followed by centrifugation and resuspension of virus containing pellet. Clarification using a fluorocarbon is achieved before small volumes are applied to cell culture or RNA extracted and virus particles multiplied by (RT)PCR (Jaykus, De Leon et al. 1996; Cromeans, Nainan et al. 1997; Mullendore, Sobsey et al. 2001).

2.3.2.4 Other extraction-concentration methods

Lesser-known extraction-concentration methods use enzymes and chemicals to digest samples, rather than the traditional mechanical homogenization approach. In one approach, 10 ml of a 10% Brewers Protease ™ solution is added to 5 g of virus containing shellfish, and allowed to digest for 2 hours at 56 °C (Legeay, Caudrelier et al. 2000). Digested oyster tissue is then clarified using a chloroform-like extraction (Legeay, Caudrelier et al. 2000). The Center for Environment, Fisheries, and Aquaculture (CEFAS) in the United Kingdom, uses a similar shellfish digestion method with the replacing proteinase K for Brewers Protease ™, and a different fluorocarbon for extraction (CEFAS, personal communication). In both the Legay and the CEFAS

methods, an emphasis is placed on rapid extraction of virus. Small sample weights (0.5-5 g) allow for similarly small sample volumes (1-10 ml), thus escaping PEG, filtration, or other concentration techniques (Legeay, Caudrelier et al. 2000).

2.3.2.5 Assay and Characterization

Viruses recovered from food samples using one of the above methods are then assayed and characterized. Specifically, the matrix of contamination, degree of clarification, sample quality, the concentration factor, and the detection limit of the assay will all weigh heavily on the ability to detect and/or quantify the virus that may be present in food samples. Two common methods for detection of enteric viruses in foods are the cell culture infectivity assay and (RT)PCR followed by some form of visualization or hybridization. Bacteriophages may be detected by (RT) PCR or through infectivity using an enrichment assay, also a single or double agar layer method. Importantly, molecular detection methods differ in sensitivity to food-based inhibitors, and the most sensitive method for virus detection in PBS or water may not always be the most robust method when detection takes place in a food-based matrix. Without elaborating further, conventional RT-PCR, nested RT-PCR, realtime RT-PCR, and infectivity assays are explained in the Methods and Materials section and critiqued in the Discussion section.

2.4 Shellfish as a Model for Food Safety Research

2.4.1 Introduction to Mollusks

Of interest to Public Health practitioners are those mollusks that: 1) are eaten by humans; 2) concentrate microbial pathogens beyond ambient levels; and 3) are important economic resources. Bivalve molluscan shellfish fulfill these three risk categories surprisingly well, and can serve as a model for applied food safety research.

By studying mollusk shells from archeological sites (ICAZ), researchers think prehistoric humans living near oceans, rivers, lakes, and other bodies of water sustained on shellfish for much of their diet. To understand the current consumption patterns for shellfish, oyster harvest yields were complied. One popular oyster speices, *C. virginica*, was commercially harvested at a rate of ~8 million bushels per year from 1980 to 1990 in Atlantic Coast and Gulf Coast waters (Kennedy, Newell et al. 1996). Commercially, the 10 million kilograms of shucked oyster meat at a value of $75 million came out of Atlantic Coast, Gulf Coast, and Canadian waters in 1989 (Kennedy, Newell et al. 1996). These are conservative estimate of US shellfish consumption given that the count represents the harvest of one species, but show the popularity of shellfish as a consumer good. Shellfish are often eaten raw or partially cooked, which coupled with the possibility for microbial contamination, leads increased risk for foodborne transmission of viral and other microbial pathogens.

2.4.2 Shellfish-borne illnesses

Although pathogenic viral contamination is a concern, and the topic of this paper, it is not the only cause of shellfish-borne illnesses. Other causes include bacterial pathogens,

chemical toxins, and allergies listed in Table 2.4.1. The severity of infection ranges from asymptomatic to acute or chronic illness, or in the case of toxicity is dose-dependent. Clinical presentation of most *Vibrios, Shigella, Campylobacter*, and Norovirus infections is gastroenteritis, while Salmonella infections cause septicemia, and hepatitis A infections result in viral hepatitis (Klontz and Rippey 1991). Chemical toxicity presents itself as four syndromes in humans (Table 2.4.1). Chemical toxins acquired from consuming shellfish actually come from marine dinoflagellates filtered as food for shellfish. The most serious risk for ingesting chemical toxins from shellfish is during a "red tide," which is characterized by a high replication rate among dinoflagellates in marine waters (Klontz and Rippey 1991). Allergic reactions also cause shellfish-borne illness in persons susceptible to IgE meditated allergies waters (Klontz and Rippey 1991).

Table 2.4.1 Shellfish-borne illnesses from organisms, chemicals, and immunological responses.

Organism	Syndrome from Chemical Toxins	Allergic Reactions
Bacteria		IgE-mediated allergies from consuming:
Salmonellas	Paralytic Shellfish Poisoning (PSP)	
S. typhi		
S. paratyphi	Neurotoxic Shellfish Poisoning (NSP)	Fish
		Mollusks
Vibrios	Diarrheic Shellfish Poisoning (DSP)	Crustacea
V. cholerae O1		
	Amnesic Shellfish Poisoning (ASP)	
V. cholerae non-O1		
V. parahaemolyticus		
V. mimicus	Modified from (Klontz and Rippey 1991)	
V. hollisae		
V. fluvialis		
V. vulnificus		
Shigella		
Campylobacter		
Virus		
Hepatitia A virus		
Norovirus		

2.4.3 Physical and Biological Properties of Oysters

To understand the mechanism by which shellfish accumulate viral and other microbial human pathogens, one can study basic physical and biological properties of shellfish. For example, oyster species including the East Coast oyster, *Crassostrea virginica,* actively captures food particles by filtering ambient waters (Kennedy, Newell et al. 1996). Adult *C. virginica* can individually filter 60 gallons or ~ 230 liters of water per day (CBF; VA-DEQ), while other sources list up to 480 liters per day for an oyster (Sobsey and Jaykus 1991). *C. virginica* feeds using two large gills, the largest organs in the body (Kennedy, Newell et al. 1996). On the way to the mouth, *C. virginica* has the ability to sort captured particles on the basis of size (Yonge 1926) and consume algae cells while rejecting inorganic particles (Newell and Jordon 1983). Once reaching the mouth, particles wrapped in mucous are pushed down the esophagus and into the stomach seen within the digestive gland. (Beninger, Le Pennec et al. 1991). Inside the stomach, enzymatic activity helps break down the cell wall of algae (Kennedy, Newell et al. 1996), and gut micro-flora aids in digestion of cellulose in as found in the Pacific oyster, *C. gigas* (Crosby and Reid 1971). Waste is then passed down the mid-gut, through the rectum and out the anus located on the dorsal side. During the feeding process, particle associated human enteric viruses or other microbial pathogens may be filtered from surrounding waters and ingested by shellfish. For this reason, past studies have investigated the ability of shellfish to accumulate, localize, and rid themselves of human enteric viruses.

2.4.4 Shellfish Microbiology Research

2.4.4.1 Accumulation of Viruses in Bivalve Mollusks

Human enteric viruses present in fecally contaminated ambient waters may come in contact with oysters and other bivalve mollusks. Active filtration increases the volume of water bivalve mollusks affect. Many laboratory trials on accumulation and depuration of viruses in bivalve molluscs began in the 1960s and continued until the late 1980s. Studies involve one or more molluscan species; often using an oyster species (*C. gigas, C. virginica, Ostrea edulis, or O. lurida*), a clam species (*Mercenaria mercenaria*) and a mussel species (*Mytilus edulis*) (Table 2.4.2). Experimental organisms were exposed to virus for between 1 hour and 1 month, although most studies last a week or less (Table 2.4.2). Methodology for laboratory aquariums differ among experimenters, with most opting for a continuous exchange of water labeled as flowing or circulating/flow-through systems, while few used static systems (Table 2.4.2).

Static systems, regardless of exposure time, concentrated <1-fold of virus in oysters (Di Girolamo, Liston et al. 1975; Jensen 1980). One research group performed laboratory experiments in a static aquarium, then again with water flow, finding more uptake was possible with water flowing (Liu, Seraichekas et al. 1966a; Liu, Seraichekas et al. 1966b). Work on flow-through or flowing systems, especially with low amounts of virus contamination, may better mimic environmental conditions. In flowing systems as much as 27-fold concentration of Poliovirus-type 1 was found in oysters, 1000-fold in clams, and 5-fold in mussels (Table 2.4.2) (Mitchell, Presnell et al. 1966; Liu, Seraichekas et al. 1966b; Gaillot, Terver et al. 1988). In a laboratory feeding experiment with HAV,

concentrations of 100-fold were found in mussels (*M. chilensis*) (Enriquez, Frosner et al. 1992), which was in sharp contrast to other work that found virus concentrations of <1-fold, 1-fold, and <1-5-fold, respectively, in exposed mussels (*M. edulis)* (Duff 1967; Croci, Flip et al. 1984; Gaillot, Terver et al. 1988).

In refining the knowledge base on virus accumulation it is also important to differentiate between shellfish consuming virus-associated particles in suspension, with virus in sediment. Landry and colleagues allowed oysters (*C. virginica*) to feed on poliovirus Lsc-2ab in resuspended or undisturbed sediment (Landry, Vaughn et al. 1983). This study virus accumulation in oysters occurred most often in virus-bound particles distributed in the water column (Landry, Vaughn et al. 1983).

Although some types of bioaccumulation in shellfish is age dependent, for example, Copper and Zinc accretes in fatty tissue of oysters (Kennedy, Newell et al. 1996), maximum or near maximum accumulation of virus took 24 hours in one species of oyster (*O. lurida*), 48 hours in another species (*C. gigas*), 24 hours in mussels (*M. edulis*), and 1-4 hours in clams (*M. mercenaria*) (Table 2.4.2) (Liu, Seraichekas et al. 1966a; Di Girolamo, Liston et al. 1975; Croci, Flip et al. 1984). Landry, Vaughn et al. (1982) postulated a *minimum concentration* of virus present in a shellfish, and when a concentration as low as 0.1 PFU/ml of poliovirus was added, an equilibrium between accumulation and depuration of virus was observed. Furthermore, concentrations <0.01 PFU virus/ml were required for detection of bioaccumulated (Landry, Vaughn et al. 1982), while future work disagreed, detecting Enteroviruses from shellfish in growing

waters below Landry and colleagues 0.01 PFU virus/ml concentration (Vaughn, Landry et al. 1978; Goyal, Gerba et al. 1979; Vaughn, Landry et al. 1979). Ultimately, improved results for the limits of accumulation in bivalve mollusks may be hampered by low concentrations of virus existing in shellfish, a lack in methods for recovery of virus at low PFU levels, and a difficulty in measuring virus load in ambient waters (Landry, Vaughn et al. 1982).

Table 2.4.2 Virus accumulation in Shellfish.

Shellfish Species	Virus type	System	Exposure Time	Concentration	Reference
Oysters					
C. virginica	P-1; CB-3	Circulating	1-7 d	1-fold	Metcalf and Stiles, 1965
C. virginica	Phage	-	-	10-fold	Hoff et al. 1965
C. virginica	P-1	Flowing	1-22 hr	10-27-fold	Mitchell et al., 1966
C. virginica	P-1	Flow-through	6-24 hr	5-18-fold	Hamblet et al., 1969
C. virginica	P-1	Flowing	0-28 d	no uptake at 5 days	Landry et al., 1982
C. virginica	P-1	Flowing	1-7 d	little uptake	Landry et al., 1983
C. virginica	P-1	Static	6-24 hr	<1-fold	Jensen 1980
C. gigas	P-1	Flowing	1 d	0.4-3.4-fold clarified virus	Hoff and Becker 1969
C. gigas	P-1	Static	2 d	<1-fold	Di Girolamo et al., 1975
C. gigas	P-2	Flowing	2-18 d	no uptake at 5 days	Landry et al., 1983
C. gigas	Cricket paralysis	Agitated	6-16 hr	1-fold	Scotti et al., 1983
O. edulis	P-3	Circulating	2 hr	1-fold	Hedstrom and Lyke 1964
O. lurida	P-1	Flowing	1 d	0.4-3.4-fold clarified virus	Hoff and Becker 1969
O. lurida	P-1	Static	2 d	<1-fold	DiGirolamo et al., 1975
Clams					
M. mercenaria	P-1	Static	1hr -6 d	30-fold in 6 days	Liu et al., 1966a
M. mercenaria	P-1	Flowing	1-48 hr	Up to 1000-fold	Liu et al., 1966b
M. mercenaria	P-1	Flowing	20-72 hr	10-100-fold	Seraichekas et al., 1968
M. mercenaria	P-1	Flowing	0-28 d	no uptake at 5 days	Landry et al., 1982
M. mercenaria	P-1	Flowing	1-7 d	little uptake	Landry et al., 1983
M. mercenaria	S-13 coliphage	Flowing	16-231 hr	2-1500-fold	Canzonier, 1971
Mussels					
M. edulis	P-3; CA-8	Flowing	18-36 hr	<1-fold	Duff, 1967
M. edulis	-	-	20 hr	1-fold	Croci et al, 1984
M. edulis	P-1	Flowing	1hr-10 d	<1-5-fold	Gaillot et al., 1988

Modified from (Sobsey and Jaykus 1991)

2.4.4.2 Localization of Viruses in Bivalve Mollusks

Some investigators go one step further than measuring the amount of virus a shellfish accumulates, and actually characterized the location of vial contamination. Basic assessment for localized viral accumulation begins with an insult to an organism (oysters, clams, or mussels) with a type of virus (HAV, Coxsackie B3, Poliovirus, etc.). Investigators then attempt to recover virus from various organs of the experimental organism. No study systematically compares multiple organisms to multiple viruses, probably due to scope and cost of multiple comparisons. Among what is known, Meinhold found the distribution of viruses in oysters (*C. virginica*) was greatest in the digestive tract concentrated 2.2×10^4 PFU per gram, next most in the gills and mantle, and somewhat present in muscle (Meinhold 1982). Other researchers found Coxsackie B3 accumulated by oysters (*C. virginica*) in highest levels in the mantle fluid, digestive diverticula and stomach (Metcalf and Stiles 1965). In oysters (*C. gigas*) Poliovirus was found to accumulate in the digestive gland (Di Girolamo, Liston et al. 1975),while in clams (*Mercenaria mercenaria*) poliovirus accumulated in the hemolymph and digestive diverticula (Liu, Seraichekas et al. 1966a). Enriquez et al. (1992) found HAV was concentrated in mussels (*Mytilus chilensis*) filtration apparatus and digestive system. Among studies measuring concentration, shellfish stomach and digestive gland retain between 50-80% of exposure virus level (Liu, Seraichekas et al. 1966a; Di Girolamo, Liston et al. 1975). Important to note, investigators report different organs (stomach, digestive gland, digestive diverticula, digestive tract, and digestive system) as being sites for contamination, but it is unlikely that so many authors dissected specimens so differently, especially because of the small size and challenging dissection which is

required. More likely is that nomenclature differences account for the incongruence in the literature.

Other ways to bridge the results in the literature are through comparing different species or using indicator microorganisms. DiGirolama, Liston et al. (1975) found oyster (*C. gigas*) accumulated and localized poliovirus Lsc-2ab similar to oyster (*C. virginica*), but captured viruses faster than *C. virginica* (Di Girolamo, Liston et al. 1975). Burkhardt and Calci (2000) used environmental samples to study the ability of oysters (*C. virginica*) to accumulate indicator microorganisms (fecal coliforms, *Escherichia coli*, *Clostridium perfringens*, and F^+ coliphage). In a year-long study, weekly measurements were taken to identify an appropriate enteric viral surrogate for oysters. The researchers found F^+ coliphage most greatly accumulated (99-fold) in oysters during late November through January (Burkhardt and Calci 2000). The finding points towards F^+ coliphages as surrogates for enteric viruses, because 78% of recorded oyster associated gastroenteritis bouts (from Gulf Coast oysters) also occurred in the months of November through January (Burkhardt and Calci 2000). F^+ coliphage may be a possible surrogate for enteric virus contamination that would to bridge the gap between different species.

2.4.4.3 Laboratory-based Depuration and Relay of Bivalve Mollusks

Depuration and relay studies are useful for three reasons: 1) to understand the physiological and biological characteristics of shellfish viral elimination; 2) to estimate the virus load in depurated shellfish and provide information used to confirm or deny the risk from human consumption; and 3) to provide information to reframe the argument for

shellfish depuration regulations. Laboratory depuration studies are important to characterize location and amounts of virus loss in a detailed and quantitative manner. Laboratory based depuration studies are often the second phase of virus accumulation studies (Hoff, Jakubowski et al. 1965; Mitchell, Presnell et al. 1966; Hamblet, Hill et al. 1969; Hoff and Becker 1969; DiGirolamo, Liston et al. 1975; Metcalf 1979; Meinhold 1982; Enriquez, Frosner et al. 1992). Studies involve one or more molluscan species; often using an oyster species (*C. gigas, C. virginica, or O. lurida*), a clam species (*M. aremaria, M. campehciensis, M. mercenaria*) and a mussel species (*M. edulis*). These studies are commonly carried out in static or flowing laboratory aquariums, but may also include natural depuration termed shellfish "relay." The relay-time to eliminate all pathogenic viruses from shellfish depends on the species being relayed, and could be influenced by other factors like water flow, temperature, seasonality, virus load in the relay water and organism, and shellfish filtration rate. As well, viral contamination may not be completely eliminated by depuration and relay alone (Sobsey and Jaykus 1991). An excellent review of the published literature on the topic of depuration and relay studies can be found in a chapter of Molluscan Shellfish Depuration by Sobsey and Jaykus (1991).

2.4.4.4 Assessing viral pathogens in shellfish

Researchers have conduct monitoring studies to enumerate the pathogenic microbial content of shellfish, to better characterized risks of infection due to consuming contaminated shellfish. Sampling schemes vary in length from single interval to multi-year trials, in the numbers of shellfish collected, the method used for detection, and type of pathogens or indicators investigated. Studies also differ in their motives, as environmental surveillance of shellfish populations, epidemiological investigations on shellfish implicated in outbreaks, or both (Le Guyader, Neill et al. 2003). Table 2.4.6 provides results from molecular surveillance of oysters, mussels, clams, and cockles for the presence of adenovirus, astrovirus, enterovirus, hepatitis A virus, norovirus, and rotavirus. Most studies only looked for enterovirus, hepatitis A virus, and norovirus in shellfish (Table 2.4.6). In some cases, half of all shellfish contained virus, with astrovirus and rotavirus in Frence mussels. Other portions of studies detected no virus in samples, with hepatitis A in oysters and mussels from France and Sweden, respectively. It is difficult to make assumptions about the prevalence of enteric viruses in shellfish among studies, because each varies in methodology, sensitivity of detection, and type of shellfish tested. For example, Formiga-Cruz and colleagues often report the % of oysters and mussels combined positive by PCR, with the level of contamination per species often not given (2002). But, it may be the case, as with Le Guyander and colleagues (2000), that viruses were detected in mussels more often than in oysters, making Formiga-Cruz data less generalizable for either species, mussels or oysters. When Formiga-Cruz and colleagues just studied mussels they found 41% contained norovirus, while results with mussels and oysters combined gave 2%, 10%, and 26% norovirus positive (2002). What

can be drawn from these results is that enteric viruses are present in some, but not all, shellfish from Europe and Japan in high enough titers as to be detected by molecular techniques. What is unknown is the number of samples not found to be positive (false positives) by PCR because of inhibition caused by the sample matrix or the sensitivity of the assay.

Table 2.4.1 Molecular surveillance of shellfish from 1996-2003.

Reference	Country	Duration Months	Organism	Shellfish Sample number	% shellfish postive for virus by (RT)PCR ADV	ASV	EV	HAV	NV	RV
Chung 1996	USA, NC	12	Oysters	31	-	-	50	17	-	-
Le Guyader 2000	France	36	Oysters	108	-	17	19	0	23	27
Le Guyader 2000	France	36	Mussels	73	-	50	45	13	35	52
Chironna 2002	Greece, Spain, Italy	11	Mussels	290	-	-	-	18	-	-
Formiga-Cruz 2002	Greece	18	oysters and mussels	144	33	-	15	4	2	-
Formiga-Cruz 2002	Spain	18	oysters and mussels	105	36	-	26	3	26	-
Formiga-Cruz 2002	Sweden	18	Mussels	54	33	-	24	0	41	-
Formiga-Cruz 2002	United Kingdom	18	oysters and mussels	173	46	-	14	1	10	-
Romalde 2002	Spain	--	Mussels	136	-	-	44	28	-	-
Romalde 2003	Spain	--	clams and cockles	28	-	-	43	25	-	-
Beuret 2003	Switzerland	3	Oysters	87	-	-	5	0	9	-
Nishida 2003	Japan	14	Oysters	191	-	-	-	-	9	-
Shieh 2003	Gulf of Mexico	18	Oysters	12	-	-	42	-	58	-

ADV = adenovirus; ASV = astrovirus; EV = enterovirus; HAV = hepatitis A virus; NV = norovirus; RV = rotavirus

2.4.5 Shellfish Harvesting, Surveillance, and Regulations

2.4.5.1 Federal and State laws

Several federal and state laws, policies, and regulations govern shellfish growing waters, harvesting, permitting, distribution, and sale. Federal laws supercede state laws, and for shellfish these include the Federal Food and Drug Cosmetic (FDC) Act, US Public Health Service Act, a federal regulation on Good Manufacturing Practices, and the Fair Packing and Labeling Act (Dressel and Snyder 1991). The FDC gives the US Food and Drug Administration (FDA) responsibility over interstate commerce of shellfish (Dressel and Snyder 1991). The FDA also has regulations for the microbiological quality of shellfish growing waters (Table 2.4.4).

The thrust of state shellfish regulation and management is by the National Shellfish Sanitation Program (NSSP), under the FDA. The NSSP is a voluntary program among 23 shellfish producing states, foreign governments, the shellfish industry, the FDA, and the National Marine Fisheries Service (Dressel and Snyder 1991). The NSSP leads an effort to administer statewide shellfish safety and sanitation programs to regulate: 1) the classification of shellfish growing areas; 2) the harvesting of shellfish; 3) Shellfish processing procedures and facilities; 4) product labeling; 5) storage, handling, and packing; 6) shellfish shipment in interstate commerce; 7) shellfish dealers; and 8) bivalve aquaculture (NSSP 1999). As noted in a survey article by Dressel and Snyder, important regulations include a compulsory certificate of origin for harvested shellfish, dealer certification in accordance with NSSP, and approved depuration techniques (1991).

Depuration is of considerable interest as a method to clear potentially contaminated oysters of harmful pathogens, thus recovering a valuable consumer product.

Not all states have a depuration program available, and must rely on relay methods to process potentially contaminated oysters. In these states especially, and in all states participating in the NSSP, understanding the quality of shellfish harvesting waters is paramount.

2.4.5.2 Indicators of viral contamination in shellfish and growing waters

Regulators in the US and Europe use fecal coliforms and/or *Escherecia coli* as bacterial indicators for pathogenic viruses, bacteria, and protozoa contamination in shellfish and shellfish growing waters (Table 2.4.4). Specifically, Europe prefers to apply microbiological standards to shellfish (100g shellfish meat) while the US tests water quality (100ml water) as a measure of possible shellfish contamination (Table 2.4.4). Furthermore, US standards rely on the assumption that shellfish accumulate pathogens in the water, thus setting low limits of surface water contamination will escape this risk (Table 2.4.4). European microbiological standards, on the other hand, allow for higher fecal coliform and/or E. coli counts because measurements are limited to shellfish.

Table 2.4.2 Regulatory standards for shellfish in USA and European Union.

Shellfish Treatment Required	US FDA Classification	Microbiological standard per 100 ml water	EU Classification *	Microbiological standard per 100 g shellfish
Not required	Approved	GM < 14 FCs and 90%< 43 FCs	Category A	All samples <230 E. coli or All samples < 300 FCs
Depuration or relay	Restricted	GM < 88 FCs and 90%< 260 FCs	Category B	90% < 4600 E.coli or 90% < 6000 FCs
Protected relaying (>2 mo)	--	--	Category C	All samples <60,000 FCs
Harvesting prohibited	--	above levels exceeded	--	Above levels exceeded

* FCs = fecal coliforms, GM = geometric mean, 90% = 90%-ile compliance.
From (Lees 2000)

To further understand the complicated nature of testing shellfish and shellfish growing water, Table 2.4.5 presents the regulatory agencies responsible for such tests. In the case of North Carolina, a typical shellfish harvesting state participating in the National Shellfish and Sanitation Program (NSSP), three different regulatory divisions are responsible for monitoring water quality and harvesting or closures. Getting statewide agencies to agree on a methodology, a venue for disseminating results, and regulatory actions based on those results is quite difficult. The NSSP helps address many of these concerns, but the ultimate health of shellfish consumers depends most on the ability of the bacterial indicator to model enteric human pathogens.

Table 2.4.3 Monitoring by activity and agency in North Carolina.

Activity	Agency
Water quality monitoring for fresh and estuarine waters	DWQ
Water quality monitoring for estuarine and ocean waters	DEH
Shoreline surveys of shellfish growing areas	DEH
Regulating shellfish harvesting	DMF
Recommending and tracking shellfish growing waters closings	DEH
Assessing loss of use for shellfish harvesting	DWQ
TMDL development	DWQ

DWQ = Division of Water Quality; TMDL = Total Maximum Daily Load
DEH = Division of Environmental Health; DMF= Division of Marine Fisheries

Importantly, a system for sanitary control rests on the gauge of the level of sanitation. Several challenges preclude testing for pathogens, and predominantly for viral pathogens, because such tests are expensive, time-consuming, and challenging. As well, not all viruses replicate in cell-culture, and detection methods may result in false-positive signals from a lack in sensitivity. Bacterial indicators for viral contamination would be preferable if such bacteria adequately modeled pathogens. But, bacterial indicators (total coliforms, fecal coliforms, and/or *E. coli*) are not correlated with enteric viral pathogens in clams (Wait, Hackney et al. 1983), mussels (Dore, Henshilwood et al. 2000; Chironna, Germinario et al. 2002), and oysters and harvest waters (Chung, Jaykus et al. 1998). A survey of shellfish depuration, Sobsey and Jaykus (Sobsey and Jaykus 1991) conclude from the findings of others that viruses are eliminated slower than bacteria (Hedstrom and Lyke 1964; Canzonier 1971; Sobsey, Carrick et al. 1980; Scotti, Fletcher et al. 1983; Cook and Ellender 1986; Power and Collins 1989), and furthermore, " bacterial elimination is not a reliable index of viral elimination during depuration and relay" (Canzonier 1971; Sobsey, Carrick et al. 1980; Scotti, Fletcher et al. 1983; Cook and Ellender 1986). These findings serve as warning for regulators who correlate bacterial indicators with pathogen load. Notably, with the current state of science few indicators are as applicable to rapid and economical detection than the host of bacterial indicators (total coliforms, fecal coliforms, *E. coli*, and Enteroccoci) currently used. Under recent scrutiny, an alternative for traditional indicators may be bacteriophages (IAWPRC 1991). Work by a British group found F-specific RNA bacteriophages strongly associated with fecal pollution in harvesting waters and in shellfish-associated outbreaks (Dore, Henshilwood et al. 2000). Later studies by the same group and other labs with sampling

sites in Greece, Spain, Sweeden, and United Kingdom found F-specific bacteriophages only valuable indicators in northern Europe (Formiga-Cruz, Allard et al. 2003). Other potential viral indicators are F+ coliphages (Dore and Lees 1995), C. perfringens (Chung et al., 1998), and bacteriophages infecting *B. fragilis* (Muniain-Mujika, Calvo et al. 2003).

2.5 Summary of Literature Review

Before the widespread use of molecular identification, bacterial pathogens comprised the majority of foodborne diseases of known etiology. Large portions of uncharacterized disease causing pathogens in foods may now be ascribed to enteric viruses, thus actualizing the suspect. It is estimated that currently around 7 of every 10 foodborne illnesses are caused by viral pathogens (Mead, Slutsker et al. 1999). Where foodborne vehicles incite viral pathogens, person-to-person contact further spreads an enteric virus among numerous secondary and tertiary cases. Through molecular epidemiology we may adequately sleuth foodborne viral outbreaks. With support from the state and federal level of government, internet-based outbreak registries such as FoodNet are able to passively track the most visible foodborne outbreaks. But, regulatory networks are decades away from validating the virological content and safety of high-risk foods like shellfish and fresh produce known to repeatedly harbor viral pathogens.

Bench-scale studies are able to inoculate enteric viral pathogens at low doses, and recover the agent after one or more days of work with moderate success. Also, viral pathogens have been inoculated in bench-scale environments, such as an aquarium, so virus fate and

accumulation may be documented. Limited environmental monitoring studies and outbreak investigations have incorporated bench-scale methods to recover and detect viral pathogens from food matrices. These efforts allude to the scale and scope that viral contamination exists within the food supply and the man-made and natural environment.

Methods to control enteric viral diseases are literally in the hands of people. Simple hand washing and hygienic practices may limit the spread of fecally associated viral pathogens. As well, proper treatment, placement, and disposal of human and animal waste will reduce environmental transmission of enteric viruses to food matrices. For capital heavy endeavors, such as wastewater treatment plants or CAFO animal waste we rely for the most part on government and the governance of commerce. These public health feats are advanced by research and the dissemination of knowledge, which is the principal reason for documents such as this.

3 Methods and Materials

3.1 Food Samples

3.1.1 Tomato sauce

Tomato sauce (TS; Harris Teeter Brand, "100% Natural Pasta Sauce, Flavored With Meat") was purchased at a local grocery store in a glass container and stored at 4°C after opening. The basic components of tomato sauce and other foods are listed in Table 3.1.1. As shown in Table 3.1.1 tomato sauce contained more per-gram total fat, saturated fat, and dietary fiber than either strawberries or oysters. The tomato sauce also contained a more complicated list of ingredients than the other two foods, including water, tomato paste, diced tomatoes, corn syrup, soybean oil, ground beef, spices (basil, oragano, and parsley), garlic and onion powder, citric acid, natural flavors (data not shown). Ground beef and spices are important components during assay, because the former material sinks in solution while the latter floats when combined with an elution buffer. Tomato sauce was homogenized at low speed for 30 seconds in a Waring Blender, and 15 mL (~30 g) was aliquoted into 50 mL capacity conical centrifuge tubes. Aliquots were stored at –20°C until assay, as what one might expect for impounded foods after a viral foodborne outbreak.

Table 3.1.1 Nutritional components in tomato sauce and strawberries, and oysters.

Component	Tomato Sauce amount per 31 g (15 mL)	Strawberries amount Per 28 g (15 mL)	Oysters Amount per 30 g (1 serving)
Total fat	0.7	0	.6
Saturated fat	0.2	0	0
Sodium	0.2	0	0.3
Total carbohydrate	5	6	1.2
Dietary fiber	0.5	0.2	0
Sugars	5	6	0
Protein	0.5	0.2	3
Vitamins and minerals	A, C, Iron, Calcium	C, Iron	A, C, Iron

3.1.2 Strawberries

Strawberries were purchased frozen, halved in syrup (SS; Birds Eye Brand). Ingredients in this food include: strawberries, invert sugar syrup, and corn syrup. Strawberry seeds may be important components during assay, because they float in elution buffers. Strawberries were homogenized at low speed for 30 seconds in a Waring Blender, and 15 mL (~30 g) was aliquoted into 50 mL capacity conical centrifuge tubes. Aliquots were stored at –20°C until assay.

3.1.3 Oysters (Crassostrea virginica)

Oysters were purchased live from a local seafood market (Durham, NC). These shellfish had been harvested in the waters of Louisiana in the Gulf of Mexico, were approximately 3-4 days old at time of purchase, and had been kept at 4°C. Shellfish were rinsed and scrubbed under tap water to remove sediment, barnacles, pseudo-feces, flatworms, and any other fowling organisms present. Oysters were shucked, drained, and the meat was recovered, and crudely homogenized in a Waring blender at low speed for 5 seconds,

processing. An approximate 10-25 gram portion of homogenized oyster tissue was aliquoted in 50 mL capacity centrifuge tubes and assayed, or stored at -20°C. No steps were taken to reduce potential background viral contamination in oysters.

3.2 Viruses

3.2.1 Hepatitis A Virus (HAV)

HAV HM175 clone p24-A was grown in confluent layers of FRhK-4 (fetal rhesus kidney) cells as described by Cromeans and colleagues (1989). FRhK-4 cells at ~90% confluency in tissue culture flasks (T-150s' contain $3x10^7$ cells were inoculate at a multiplicity of infection of 0.1 to 0.01with 1-2 ml of 3×10^5 to 3×10^6 PFU HAV. Flasks were incubated at 37°C for 60 min, then 30-50 ml serum-free maintenance media was added and incubated 1 wk at 37°C or until 95% of cell monolayer is infected.

Cells were harvest by 3X freeze-thaw episodes, then homogenized on high speed for 2 min in an Omni Mixer with half to equal volumes of a fluorocarbon (Vertrel XF), centrifuged at 4800 RPM in RC3B centrifuge for 20 min. The virus rich supernatant was recovered and labeled crude virus stock. HAV used in spiking experiments was from crude virus stock or dilutions made in phosphate buffered saline (PBS; pH 7.2). Virus stock was titered using cell culture technique reported by Cromeans and colleagues (1989). Briefly, FRhK-4 cells were grown to ~90% confluency in 60 mm petri dishes, growth media aspirated from dishes using sterile technique, and 200ul of sample was added. Dishes were incubated at 37°C in a CO_2 incubator for 60 min with manual gentle rocking every 15 min. A 1% agarose overly medium containing 1% agarose (Acros Organics, New Jersey), 2X Eagles MEM (Linberger Tissue Culture Facility, Chapel Hill,

NC), and media supplements were made, then 5-7 ml of overlay medium were added onto dishes. Dishes were placed in a CO_2 incubator for 7 days at 37°C. On the seventh day a second 1% agarose overlay as above, but with a 1% neutral red dye to help visualize plaques. Plaques were visualized, counted, and recorded as plaque forming units (PFUs) per ml. HAV was also titered using reverse-transcription polymerase chain reaction (RT-PCR) to an endpoint dilution as visualized by gel electrophoresis, and reported as RT-PCR units.

3.2.2 Coliphage MS2

Coliphage MS2 (ATCC 15597-B1) was enumerated using a double-agar layer (DAL) method, in which molten 0.8% tryptic soy agar is combined with the sample and log-phase host bacteria (*E. coli* C3000), then poured onto a petri dish containing 15 ml of solidified 1.4% tryptic soy agar (EPA method 1602; Sobsey, Schwab et al. 1990; Sobsey, Battigelli et al. 1995). Plates were inverted and placed in a 37°C incubator overnight. Plaques, or clear zones of lysis in the host lawn, were enumerated and recorded as plaque forming units (PFUs).

3.2.3 Seeded food samples with laboratory virus

Tomato sauce (15 mL) strawberry sauce (15 mL), and oysters (25 g of whole oyster or and equivilant weight of dissected oysters) were seeded with small volumes of HAV and/or MS2 (0.1 mL). Seeded samples were incubated at room temperature for 15 min to allow for virus adsorption (Cromeans et al., 1997, Mullendor et al., 2001). Work was performed in a laminar flow hood using autoclaved or UV sterilized plasticware, pipettes, and glassware.

3.3 Virus concentration and purification

3.3.1 Eluants

Buffered protein solutions (eluants) were made from standard dry-goods reagents purchased from chemical suppliers and de-ionized water and used in elution and acid-adsorption elution experiments. Eluants include 10% tryptose phosphate broth/ 0.05 M glycine (pH 8 and 9.5), 0.5 M gylycene/0.1% Tween-80 (pH8) buffer, 0.05 M glycine-0.15 M NaCl, 0.5 M threonine-0.15 M NaCl, and 3% beef extract buffer. Eluants were prepared in 1L or 0.5L glass containers (Pyrex), pH adjusted using 5 M NaCl and 5 M HCl, then autoclaved and stored at 4°C.

3.3.2 Polyethylene glycol (PEG) precipitation

Viruses in food eluates and extracts were precipitated with PEG. Dry PEG (Sigma, St. Louis, MO) and NaCl were added to 30-250 ml sample eluates to a final concentration of 8% PEG and 0.3 M NaCl. Samples were magnetically mixed for 15-30 min at room temperature, until the PEG and salt went into solution. Samples with small volumes (~30-50 ml) were stored for 2-4 hours at 4°C, or for larger samples, overnight at 4°C to facilitate precipitation. After precipitation, samples were centrifuged at 7,000-15,000 xg for 15-30 min. The supernatant was discarded and the virus-containing pellets were resuspended in small volumes of PBS, water or other buffers.

3.3.3 Small volume RNA extraction

Small volumes of HAV were extracted with QIAmp viral RNA mini kit (Valencia, Ca). The mini kit extracts RNA using a proprietary guanidinium reagent. RNA is extracted from viruses in samples by utilizing the selective binding properties of silica-gel-based

membrane contained within each spin column. The sample is first lysed under highly denaturing conditions to release RNA, followed by binding of the RNA to the membrane, and inactivation of any enzymes (*e.g.,* RNases) that may degrade the liberated RNA. During subsequent steps, the bound RNA is retained on the membrane while contaminants and impurities are washed away. Then, the RNA is eluted from the membrane and is recovered in a small volume of water (about 50-60 uL). In some experiments this was substituted with a guanadinium isothiocinate (GuSCN) lysis buffer (Jothikumar, N., Sobsey, M.D., and T.L. Cromeans, personal communication; unpublished data) along with various ethanol-based wash solutions. Using the commercial kits, samples of 140 uL were chemically extracted and passed through the silica gel spin column with successive washing steps, according to manufacturers directions. In some experiments columns were reloaded with additional 140 uL volumes (S. Shumoski, Qiagen Molecular Diagnostics, personal communication). RNA bound to the spin column was eluted with 50 uL of TE buffer (containing 5 mM Tris-0.5 mM EDTA, pH 8.0, Ambion, Austin, TX). Eluted virus nucleic acids were stored in aliquots at –80°C, processed directly, and/or alcohol concentrated.

3.3.4 Large volume RNA extraction

The QIAmp Viral RNA Mini Kit (Qiagen, Valencia, CA) only provides 8 mL of lysis buffer and as a result was cost-prohibitive for resuspending multiple large-volume PEG pellets. Larger Qiagen Midi column kits (RNeasy Kit, Qiagen, Valencia, CA) contain adequate lysis buffer, but proprietary components limit the ability to modify reaction conditions to optimize RNA extraction from spin columns. A GuSCN lysis buffer was created with similar properties to the Qiagen lysis buffer was used in experiments

involving large volume RNA extraction. GuSCN lysis buffer has shown to work similarly to Qiagen's lysis buffer and was used in large volume RNA extraction (Jothikumar *et al.*, 2003.

GuSCN lysis buffer contained120 grams of guanidine thiocyanate (GuSCN) (Boehringer Mannheim, Indianapolis, IN) in 100 mL of TE buffer (containing 5 mM Tris-0.5 mM EDTA, pH 8.0, Ambion, Austin, TX) prepared with nuclease free water (Ambion, Austin, TX). After the GuSCN dissolved, 11 mL of 5 M sodium chloride (Ambion, Austin, TX) and 11 mL of 3 M sodium acetate pH 5.5 (Ambion, Austin, TX) were added. Carrier RNA (Poly A from Sigma, St Louis, MO) was added to a final concentration of 20 µg/mL. Like the Qiagen reagent, GuSCN lysis buffer acts under highly denaturing conditions to release RNA, while simultaneously inactivating any enzymes (e.g., RNases) that may degrade the liberated RNA.

In the present study, three to five mL of GuSCN lysis buffer was added to resuspend PEG pelleted material containing virus or other samples. These mixtures were briefly vortexed and incubated at room temperature for 15 min to allow for virus capsid lysis. An equal volume of absolute molecular grade ethanol (Ambion, Austin, TX) was added to GuSCN virus lysate, vortexed briefly, and then passed through Qiagen Midi columns (RNeasy Kit, Qiagen, Valencia, CA) by centrifugation at spin speeds suggested by the manufacturer. Midi columns were loaded between 1 and 3 times with virus lysate. Virus and food material binding to Midi column was washed with a solution of 1 ml GuSCN lysis buffer, 1 mL nuclease free water (Ambion, Austin, TX), and 2 mL absolute

molecular grade ethanol (Ambion, Austin, TX), washed with a solution of 75% molecular grade ethanol (Ambion, Austin, TX), washed a third time with the aforementioned GuSCN/water/ethanol solution, and subsequently eluted off the column with 200 µL of TE buffer (10 uM Tris-1uM EDTA, pH 8.0; Promega, Madison, WI) (Vinje, personal communication; unpublished data). GuSCN waste was stored in a 1 L container with 13.6 grams NaOH added, as to preclude the formation of toxic cyanide gas.

3.3.5 Alcohol nucleic acid precipitation

RNA extracted samples larger than 50 µL were further concentrated with one of two types of alcohol nucleic acid precipitation, isopropanol or alcohol (Table 3.3.1). These methods concentrate 200 µL of RNA tenfold, thus increasing the sensitivity of detection tenfold. Alcohol nucleic acid precipitation involves the addition of 5 M ammonium acetate, alcohol, and a non-reactive marker (a blue colored starch) to a micro-eppendorf tube containing 200 µL of RNA eluted from a Qiagen Midi column (RNeasy Kit, Qiagen, Valencia, CA). The alcohol precipitation mixture was chilled for 30 to 60 min at –20 °C then centrifuged to pellet the RNA. The pellet was washed with cold absolute molecular grade ethanol (Ambion, Austin, TX), recentrifuged, and air dried, resulting in a final pellet of RNA that was resuspended in a small volume (10-20 µL) of TE buffer (5 mM Tris pH 8.0, 0.5 mM EDTA pH 8.0) or nuclease free water (Ambion, Austin, TX).

Table 3.3.1 Precipitation of viral RNA

Isopropanol		Ethanol	
1. Add 1/10th volume of 5 M ammonium acetate to RNA; mix.	6. Remove supernatant and discard.	1. Add 1/10th volume of 5 M Ammonium acetate to RNA. Mix.	6. Remove supernatant and discard.
2. Add equal volume of isopropanol; mix.	7. Wash with 500 uL of 75% ice cold ethanol.	2. Add 2.5 volumes of cold 75% molecular grade ethanol; mix.	7. Wash with 500 uL of 75% ice cold ethanol.
3. Add 1 uL of glycoblue; mix.	8. Centrifuge at 14,000 RPM for 5 min. at 4°C.	3. Add 1 uL of glycoblue; mix.	8. Centrifuge at 14,000 RPM for 5 min. at 4°C.
4. Keep at -20°C for 30-60 min.	9. Remove supernatant and air dry pellet.	4. Keep at -20°C for 30-60 min.	9. Remove supernatant and air dry pellet.
5. Centrifuge at 14,000 RPM for 15 min at 4°C.	10. Resuspend pellet in desired volume of TE or nuclease-free water.	5. Centrifuge at 14,000 RPM for 15 min at 4°C.	10. Resuspend pellet in desired volume of TE or nuclease-free water.

3.4 Molecular Detection Methods

3.4.1 Conventional reverse-transcription polymerase chain reaction

Conventional RT-PCR was carried out using reagents from Qiagen OneStep RT-PCR kit (Valencia, CA) following manufacturers instructions. The master mix per-reaction included 5 uL of 5X Qiagen RT-PCR buffer, 1 uL dNTP mix to a final concentration of 0.4mM, 0.5 uL of forward and reverse primers (Table 3.4.1) to a final concentration of 50 pmol/uL, 1 uL of Qiagen enzyme mix containing Taq polymerase, 0.5 uL containing 20 U of RNase inhibitor, 14 uL of RNase free water, and 2.5 uL of RNA template, giving a single reaction total volume of 25 uL. Primers for HAV were selected from published work by Schwab and colleagues (1995) (Table 3.4.1). Primers amplified a 192 bp length

segment of the HAV genome connecting the VP1 and VP3 capsid proteins (Schwab et al., 1995). RT-PCR reaction was carried out in a MJ Research PTC-200 Peltier Theromcycler (MJ Research, Waltham, MA) with the following RT and PCR cycles and temperatures: (RT) 42°C for 30 min, 95°C for 15 min, (PCR x 40 cycles) 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min.

Table 3.4.1 Primers used in conventional RT-PCR assays.

Virus	Designation	Sequence (5'-3')	Amplicon Length (bp)	Reference
HAV	HAV fwd	CAGCACATCAGAAAGGTGAG	192	Schwab, 1995
	HAV rvs	CTCCAGAATCATCTCCAAC		

Twenty percent of amplified PCR products were separated by weight on 2% agarose gels stained with ethidium bromide, and visualized under UV light. In small tubes or in 96 well cell culture tubes, 10 ul of amplicon was added to 2 ul of 6X Loading buffer (Promega, Madison, WI), and 5 ul of DNA ladder (Promega, Maidson, WI) was added to 1 ul of 6X loading buffer (Promega, Madison, WI). Cooled agarose gels were submerged in 1X TAE in a gel box (Fotodyne Inc., Hartland, WI), dye colored samples and 100 bp DNA ladder added to appropriate the wells, and 125 volts were passed through the machine for 30-45 minutes (Foto/Force 300, Fotodyne Inc., Hartland, WI). After stained amplicon had spread an appropriate length, gels were visualized by UV, photographed, and the picture stored to floppy disc (Gel print 2000i, Biophotonics, Pittsfield, MA).

3.4.2 Nested reverse transcription polymerase chain reaction

A nested RT-PCR assay, consisting of two rounds of amplification, was used to multiply HAV RNA for further visualization by gel electrophoresis. First round RT-PCR used the one-tube, One-Step kit (Qiagen, Valencia, CA), following manufacturers instructions.

Reagents include 5X Qiagen RT-PCR Buffer (1X final conc.), dNTP mix (0.4mM final conc.), enzyme mix (1ul per rxn), RNase inhibitor (20 U), primers (50 pmol final conc.), and RNase-free water. First round primers spanned the conserved VP1-VP3 capsid region of the HAV genome, were 319 bp long, and came from a published source (Hutin, Pool et al. 1999) (Table 3.4.2). Two and a half µl of RNA template was added for a total reaction volume of 25 ul. RT-PCR was carried out in a MJ Research PTC-200 Peltier Theromcycler (MJ Research, Waltham, MA). RT steps included a cycle at 42°C for 30 min, and heating at 95°C for 15 min. Further steps included 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final cycle at 72°C for 10 min.

Second round "nested" PCR used HotStar Taq mastermix (Qiagen, Valencia, CA), following the manufacturer's instructions. Reagents include HotStar Taq mastermixprimers (2x), primers (50 pmol final conc.), and RNase-free water. The second round primers spanned 243 bp across the conserved VP1-VP3 capsid region of the HAV genome and were internal to the first round primers (Hutin, Pool et al. 1999) (Table 3.4.2). A laboratory cabinet was dedicated for use with nested reactions. One ul of 1st round RT-PCR template was added for a total reaction volume of 25 ul. PCR was carried out in a MJ Research PTC-200 Peltier Theromcycler (MJ Research, Waltham, MA). Steps included heating at 95°C for 15 min, followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, with a final cycle at 72°C for 10 min.

Table 3.4.2 Primers for Hepatitis A virus, nested RT-PCR assay

Virus	Designation	Sequence (5' → 3')	Amplicon length (bp)	Reference
HAV 1st round	External HAV fwd	GTTAATGTTTATCTTTCAGCAAT	319	Hutin, 1999
	External HAV rvs	GATCTGATGTATGTCTGGATTCT		
HAV 2nd round	Internal HAV fwd	GCTCCTCTTTATCATGCTATGGAT	243	Hutin, 1999
	Internal HAV rvs	CAGGAAATGTCTCAGGTACTTTCT		

Forty percent of amplified PCR products were separated by weight on 2% agarose gels stained with ethidium bromide, electrophoresed, and visualized under UV light. Both first and second-round products, and appropriate positive and negative controls were run side-by-side on gels.

3.4.3 Real-Time RT-PCR amplification and detection

Real-time RT-PCR was carried out in 25 µl reaction tubes using Smart Cycler (Cepheid, Sunnyvale, CA) technology. The Smart Cycler (Cepheid, Sunnyvale, CA) uses a ceramic heating plate and a high-efficiency fan to rapidly change the ambient temperature in each reaction vessel. Reaction wells contain high intensity light-emitting diodes (LEDs) to stimulate excitation and emission of four types of fluorescent-labeled dyes used as hybridization probes within a reaction. A silicon photodetector measures the fluorescence and links to a computer whose software (Smart Cycler version 1.2f, Cepheid, Sunnyvale, CA) interprets the signal. The QuantiTect Probe RT-PCR kit (Qiagen, Valencia, CA) was used for HAV real-time RT-PCR assays. Reaction components included 12.5 µL of 2x QuantiTect Probe RT-PCR Master Mix containing HotStarTaq DNA polymerase to a final concentration of 1x; 2 U QuantiTect RT Mix to a final concentration of 2U/rxn; 0.5 µl of forward primer to a final concentration of 0.4

μM; 0.5 μl of reverse primer to a final concentration of 0.4 μM; a fluorescent-labeled probe to a final concentration between 0.1 to 0.25 μM, and nuclease free water. HAV probe had a ROX or FAM fluorescent label conjugated at the 5 end and a Black Hole Quencher conjugated at the 3 end (Table 3.4.3). The probe detected HAV's 89-bp RNA-dependant RNA polymerase gene fragment (Jothikumar, N., Sobsey, M.D., and T.L. Cromeans, personal communication; unpublished material). Primers and probe for HAV real-time RT-PCR are based on HAV strain HM175 from GENBANK as reference for the nucleotide positions listed. They were synthesized at the biotechnology core facility at CDC-Atlanta, for which sequences are listed in Table 3.4.3.

Table 3.4.3 Oligonucleotide primer/probe sequences for HAV in Real-time RT-PCR.

Primers or Probe	Primer or Probe	Sequence (5 → 3)	Tm (°C)	Position
Forward	HAV-F	5 GGTAGGCTACGGGTGAAAC -'3	59.4	392-410
Reverse	HAV-R	5 AACAACTCACCAATATCCGC -'3	59.8	480-461
Probe	HAVRox or HAVFam	5 CTTAGGCTAATACTTCTATGAAGAGATGC-3	61.4	413-441

Primers and probe from (Jothikumar, et al, 2004). Sequence of HAV HM175 from GENBANK used as reference for the nucleotide positions listed.

The master mix containing the reaction components listed above was aliquoted in 20-24 μl portions into Smart Cycler tubes (Cepheid, Sunnyvale, CA) resting in the manufacturer's pre-chilled cooling block. To the reaction mixture 1-5 μl RNA template was added, or an equivalent amount of nuclease free water as a negative control to a total volume of 25 μl. Reaction tubes were firmly closed, as distinguished by a characteristic "clicking" sound, signifying the hermetic seal and necessary pressurization of its

contents. Smart Cycler tubes were briefly centrifuged according to the manufacturer's instructions to remove air bubbles inside the reaction matrix. Visual inspection of the tubes and gentle agitation of the centrifuge tubes was required to further remove trapped air bubbles. Reaction conditions for real-time RT-PCR of HAV included reverse transcription for 30 min at 50 °C, then 15 min at 95 °C to activate Taq Polymerase, and finally amplification for 45 cycles of 10 sec at 95 °C, 20 sec at 55 °C, and 15 sec at 72 °C (Jothikumar, N., Sobsey, M.D., and T.L. Cromeans, personal communication; unpublished material). Detection of fluorescing ROX or FAM labeled probes occurred during the 55 °C stage in each cycle on, as visualized by optical channel 4 (605-800 nm). Positive signals were detected in samples with a significant increase in fluorescence above a background threshold—as a sample's cycle threshold (Ct). Unknown positive samples were compared to standard curves from ten-fold dilutions of a known quantity of like virus, giving a quantitative analysis of unknowns.

3.5 Statistical Analysis

Statistical analysis of data was performed on InStat version 3.06 (Graphpad, Software Inc.). Non-parametric analysis of variance was performed using the Mann-Whitney Test for two unpaired groups of data and the Kruskal-Wallis Test for three or more unpaired groups of data. The P value, statistical test, and statistical significance are reported for all data analyzed by InStat (Graphpad, Software Inc.). Excel (Microsoft® Office Excel 2003) was used to generate means and standard deviations found in tables and figures.

4 Results

4.1 Overview

Although extensive literature exists on methods for the recovery and molecular detection of enteric viruses in shellfish, methods to achieve similar goals in internally contaminated produce, sauces, and mixed foods are not well characterized. Only two peer-reviewed journal articles have been published on methods for recovery and molecular detection of enteric viruses from internally contaminated sauces ((Hewitt and Greening 2004) and meat (Leggitt and Jaykus, 2000). No papers are available in peer-review journals regarding recovery and molecular detection of enteric viruses from any other internally contaminated food, although outbreaks have occurred in produce, fresh and frozen fruits, breads, and prepared foods or mixed foods, which justify such research (Gaulin, Frigon et al. 1999; Lopman, Reacher et al. 2003). Furthermore, the Centers for Disease Control and Prevention recommends that state public health laboratories and other agencies not attempt to recover enteric viruses (specifically Norovirus) from contaminated foods citing, "methods are not sufficiently developed to be routinely applied" (MMWR, 2001).

Based on a review of the published literature, two candidate methods were selected for study of their ability to recover viruses from food matrices. The two are acid-adsorption elution type methods and alkaline elution type methods. The following sections provide results and interpretation of the development of and efficiencies in critical steps in both

each method type. After developing the methods based on the results of qualitative and quantitative studies, the compatibility of the sample matrix with a detection method was then investigated, and finally results are presented on the virus recovery efficiency and detection limit of each developed method.

4.2 Alkaline Elution Methods

Alkaline elution methods attempt to alter the virus-food relationship through manipulation of conditions that promote freeing of viruses from food solids. Typically, slightly to moderately alkaline (pH 7.5- pH 9.5) buffered solutions are applied to the food-virus matrix to reach or exceed in the electronegative range of surface charge (potential) the isoelectric point of a virus, causing repulsion of the negative charge on the virus and food particles, and thereby releasing the virus into the eluting solution. . The elution medium also may contain chemicals that promote virus elution from food solids by competing with the viruses fro adsorption sites on food particles. Such chemicals include beef extract and various amino acids.

With a dearth of information on elution methods for internally contaminated foods, investigation into recovery methods began with several simple approaches. Early experiments presented herein with low virus recovery, or poor virus detection are reported to inform the reader about the systematic progression towards more robust methods developed. There were several types of elution experiments performed: (1) develop and modify existing methods to recover HAV and/or MS2 from produce and sauces; (2) evaluate the ability of previously reported eluents to recover HAV and MS2 from oysters; (3) evaluate the compatibility of samples with detection of HAV in cell

culture and conventional RT-PCR; and (4) identify the lower detection limit of HAV seeded initially in sauces and oysters using a developed elution method.

4.2.1 Recovery of viruses from blended strawberries and tomato sauce using a simple alkaline elution and PEG precipitation method

To determine if infectious HAV could be recovered and detected directly in strawberry sauce, experiments were conducted in which 1 mL portions of strawberry sauce were seeded with approximately 1 x 10^6 PFU HAV. Samples were vortexed for 10 seconds, and then allowed to sit at room temperature for 15 minutes for virus to adsorb to the strawberry sauce. Samples were centrifuged in microeppendorf tubes at 14,000 rpm for 5 minutes at room temperature, and the supernatant was recovered by pipette, diluted with PBS, and assayed for infectious virus by cell culture. The concentration of infectious HAV in the control strawberry sample was 1600 (3.2 $\log_{10}$) PFU/ml. Hence, less than 1% of the infectious virus was recovered from this sample (data not shown). It is unclear if this was due to loss in the recovery method or to the conditions of the strawberry material may have inactivated the virus or interfered with its detection by infectivity.

In an attempt to improve recovery of viruses from strawberry sauce, 1 ml portions of strawberry sauce were seeded with about 1 x 10^6 PFU HAV and then eluted with 1 ml of 0.5 M glycine / 0.1% Tween-80 (pH 8). This elution medium was chosen because it had been previously found to be effective in eluting viruses from the surfaces of produce and meat (ground beef and poultry; Hsu et al., 2002). Following centrifugation, the supernatant eluant was removed (approximately 0.8 ml each), extracted with an equal volume of chloroform, then concentrated and washed with distilled deionized water, in

Centricon-100 ultrafilters (Casteel, 2001). Larger samples of strawberry sauce (10 ml) also were seeded with about 1 x 10^6 PFU HAV, and eluted with 10 mL 0.5 M Glycine/ 0.1% Tween 80 (pH 8.0), which gave resulting supernatants from the 10 ml portions that were precipitated with PEG as described in Materials and Methods. The PEG pellets were resuspended in 0.2 ml of 0.5 M glycine / 0.1% Tween-80 (pH 8), extracted with a half volume of chloroform, and analyzed for the presence of HAV via heat release RT-PCR (10 ul sample volumes) or by silica-based spin column RNA extraction (0.14 ml sample volumes) (Qiagen, Valencia, CA) followed by RT-PCR. HAV was not detected using RT-PCR in PEG concentrated samples or in samples from centrifugal ultrafiltration with a Centricon (results not shown). Even though 1 x 10^6 PFU HAV was inoculated, poor recovery and/or the presence of food-based inhibitors to RT-PCR reactions is the likely explanation of the a failure to detect HAV RNA in these samples. Based on the theoretical detection limit of the assay which was 50,000 PFU, and in the absence of positive RT-PCR detection in samples, one can assume that <100 PFU/mL of sample was recovered.

In the previous experiments attempting to recover HAV from strawberry sauce, the pH of the eluant-strawberry mixture was not determined or adjusted to account for the change in pH of the eluate that occurs after addition to the strawberry sauce. Hence, we measured the pH of 15 mL portions of strawberry sauce mixed with 25 mL of 0.5 mL glycine / 0.1% Tween-80 (pH 8), which was found to be acidic (pH 4.4). The addition of 0.6 mL of 5M NaOH changed the pH of the mixture to pH 8.5. To determine the recovery efficiency of viruses from strawberry sauce using glycine eluate with pH adjustment, coliphage MS2 was seeded in the strawberry sauce and processed using the procedure in

Figure 4.2.1, up to step 3).

The eluates were assayed for the presence of MS2 by the double agar layer technique. Fifteen (15) mL samples of strawberry sauce seeded with 4 x 10^9 PFU or 4 x 10^{10} PFU, were mixed with 25 mL of glycine, and the mixture was adjusted to pH 8.5 with 5M NaOH. Using pH adjustment of the strawberry sauce, one hundred percent (100%) of seeded MS2 was recovered from strawberry sauce in both trials (data not shown).

Figure 4.2.1 Protocol for recovery of HAV and MS2 from strawberry sauce using an amino acid elution procedure with pH adjustment

1. Add 25 ml of glycine eluant to 15 ml strawberry sauce; adjust to pH 8.5 with 5M NaOH
2. Shake at 100 RPM for 30 minutes on rotary shaking platform
3. Centrifuge at 7000*xg* for 20 minutes to remove solids; recover supernatant
4. Perform PEG precipitation on supernatant
5. Elute PEG pellet and other pelleted material with 15 mL of glycine eluate
6. Centrifuge resuspended material at 7000xg for 20 minutes
7. Withdraw supernatant; perform PEG precipitation
8. Resuspend pellet in 2 ml of PBS
9. Assay

This procedure was then applied to recover HAV from strawberry sauce. Strawberry homogenate samples (15 ml) were seeded with about 2 x 10^4 or 2 x 10^5 RTPCR units of HAV for final concentrations of 1.3 x 10^3 and 1.3 x 10^4 RTPCRU/ml, respectively. HAV recovery was attempted by the protocol in Figure 4.2.1. The final sample was divided into 1 ml portions and transferred into Eppendorf microfuge tubes and spun at full speed for 5 minutes to separate any additional particulate matter before portions (0.14 ml) were RNA extracted and 10 µl of extracted template (in a 100 µl total PCR reaction mixture

volume) was amplified using a two-step RT-PCR assay (Casteel, 2001). The lower detection limit of the assay was 1 PFU in 10 ul of RNA extracted template for RT-PCR. Assuming 100% recovery, there should be 100 RT-PCR units (or 4.3 PFU) detected in the sample seeded with about 2 x 10^4 RTPCR units of HAV, and 1,000 RT-PCR units (or 43 PFU) in the sample seeded with 2 x 10^5 RTPCR units HAV. Using this procedure, HAV RNA was detected in both strawberry homogenate samples (Figure 4.2.2), but endpoint dilutions of positive samples were not performed to establish a percent recovery.

Figure 4.2.2 RT-PCR of HAV recovered from strawberry homogenates

Lane 1- 10 µl of RNA extracted from strawberry sauce sample seeded with 200,000 RTPCRU of HAV

Lane 2- 10 µl of RNA extracted from strawberry sauce sample seeded with 20,000 RTPCRU of HAV

Lane 3- empty

Lane 4- positive control HAV; *192 bp amplicon (Schwab, 1995)*

Lane 5- 100 bp ladder

These results indicates that HAV RNA can be recovered and detected by RT-PCR from 15-ml samples of strawberry sauce containing high levels of viruses (1,000-10,000 RT-PCR units. This is a relatively high level of viruses and probably higher than the levels of virus contamination likely to be encountered in some, if not many fecally contaminated samples. Therefore, later in this report we will present the results of experiments in which lower levels of viruses were seeded into strawberry sauce and subjected to recovery and RT-PCR detection.

Experiments were also conducted to recover HAV and other viruses from tomato sauce. In the first set of experiments involving recovery of viruses from tomato sauce, 2×10^4 or 2×10^5 RTPCR units of HAV were seeded in 15 ml of tomato sauce for final concentrations of 1.3×10^3 and 1.3×10^4 RTPCRU/ml. The procedure in figure 4.2.3 was then used to process the tomato sauce samples and recover the HAV.

Figure 4.2.3 Protocol for elution of HAV and other viruses from tomato sauce

1. In 50 mL conical centrifuge tube: seed tomato sauce (15 ml) with virus; vortex briefly; allow virus to adsorb to sauce for 15 minutes at room temperature

2. Add 25 ml of glycine eluant to tomato sauce; adjust pH to 8.5 with 5M NaOH

3. Shake at 100 RPM for 30 minutes on rotary shaking platform

4. Centrifuge at 7000x*g* for 20 minutes to remove solids; adjust supernatant pH to 7.2

5. Perform PEG precipitation on supernatant (~35 ml)

6. Elute PEG pellet and other pelleted material with 15 ml of 0.5 M Glycine/0.1% Tween 80 (pH 8.0)

7. Centrifuge resuspended material at 7000xg for 20 minutes to remove solids

8. Perform PEG precipitation on supernatant (~15 ml)

9. Resuspend PEG pellet in 2 ml of PBS

10. Transfer 1 ml to microcentrifuge tube; spin at full speed 1 minute; recover supernatant

11. Extract RNA from supernatant

Figure 4.2.4 RT-PCR of HAV recovered from tomato sauce samples

Lane 1- 100 bp ladder

Lanes. 2-9, samples from previous experiments

Lane 11, 10 uL of RNA extract from tomato sauce seeded with 20,000 RTPCRU of HAV

Lane 12, 10 uL of RNA extract from tomato sauce seeded with 200,000 RTPCRU of HAV

192 bp amplicon (Schwab, 1995).

The lower detection limit of the assay was 1 PFU in 10 ul of RNA extracted template for RT-PCR. Assuming 100% recovery, there should be 100 RT-PCR units (or 4.3 PFU) detected in the sample seeded with about 2 x 10^4 RTPCR units of HAV, and 1,000 RT-PCR units (or 43 PFU) in the sample seeded with 2 x 10^5 RTPCR units HAV. As shown in Figure 4.2.4, HAV RNA was detected in tomato sauce samples seeded with both 2 x 10^4 and 2 x 10^5 RTPCR units, although endpoint dilutions of positive samples were not performed to establish a percent recovery. Relatively large quantities of HAV were recovered from seeded tomato sauce and strawberry sauce by the elution-concentration methods (by protocols in Figures 4.2.1 and 4.2.2) and detected by RT-PCR followed by visualization of amplicons by gel electrophoresis. In contrast to previous elution protocols that failed to recover and/or detect HAV RNA, it appears that these latter protocols (Figures 4.2.1 and 4.2.2) improved virus recovery.

4.2.2 Recovery of viruses from oyster tissue using a simple direct elution and PEG precipitation method

Although the work below (on oysters) is not a natural progression from elution experiments on produce and sauces, it does provide information for which to compare the elution methods and eluents described in the present study to the +35 years of scientific information gathered on recovery of enteric viruses from shellfish. Direct elution methods for shellfish were developed as early as 1968 (Herman and Cliver), and were later refined by others (Lewis and Metcalf; 1988; Lees et al., 1994) as surveyed by Shieh and colleagues (2000). Oysters and other bivalve shellfish, such as mussels and clams, are important for public health research because they bioaccumulate enteric viruses through filter feeding (Metcalf and Styles, 1965; Mitchell et al., 1966), and have been

associated with viral foodborne outbreaks from HAV and Noroviruses (Desenclose 1991; Xu 1992; Le Guyader et al., 1996; Shieh et al., 2000). Because shellfish are high risk foods for viral contamination and recovery and detection of enteric viruses in shellfish is challenging, these organisms serve as a prototype for enteric viral recovery and detection from other foods.

A direct elution approach was investigated in the present study after published reports showed elution may recover more enteric viruses than another approach, acid-adsorption elution. In one study, Shieh and colleagues (1999) recovered more initially seeded Poliovirus by a direct elution (79%) than by acid-adsorption elution (59%). Increased recovery during the critical elution step trickled down throughout the direct elution method, giving 59-53% recovery after a PEG concentration step, while acid-adsorption elution method only recovered 38-30% (Shieh et al., 1999). In this study, a simple direct elution approach was developed by combining homogenization methods, elution methods, and PEG methods previously described by other investigators (Lewis and Metcalf, 1988, Shieh et al., 1999). The simple direct elution approach is shown in Figure 4.2.6 below, and was evaluated for its ability to recover infectious coliphage MS2 and HAV from 25 grams of oyster tissue. Four different eluents 10% tryptose phosphate broth / 0.05M glycine at both pH 8 and 9.5, 0.5M glycine / 0.1% Tween-80 (pH 8), and 3% beef extract / 0.3M NaCl (pH unadjusted) were employed in experiments to recover MS2 and HAV. These eluents were chosen as candidate eluents based on their ability to recover high quantities of rotavirus SA11 from homogenized oysters (Lewis and Metcalf, 1988). MS2 and HAV were detected by infectivity assay, because it was hypothesized

that simple direct elution may contain high levels of enzymatic inhibitors to RT-PCR. Significant inhibition was confirmed when a more rigorous multiple elution and PEG concentration method was assayed by conventional RT-PCR, as shown in section 4.2.4. The results from simple direct elution experiments are shown in Tables 4.2.2-4.

Figure 4.2.5 Direct Elution and PEG precipitation method for the recovery of viruses from oysters

1. Add 175 mL of eluent (TPB pH 8 or 9.5, GLY, or BEX) to oyster sample

2. Homogenize / blend at high speed for 30 seconds in Waring blender

3. Let blender vessel sit for 10 minutes for foam to dissipate

4. Pour homogenate into 500 mL centrifuge bottle; recover remaining foam or liquid in blender vessel with pipet

5. Centrifuge at 2000*xg* for 20 minutes

6. Recover supernatant

7. Perform PEG procedure on remaining supernatant; add 8% PEG and 0.3 M NaCl

8. Resuspend PEG pellet in 10-20 mL of PBS

TPB pH 8 = 10% tryptose phosphate broth / 0.05M glycine at pH 8
TPB pH 9.5 = 10% tryptose phosphate broth / 0.05M glycine at pH 9.5
GLY = 0.5M glycine / 0.1% Tween-80 (pH 8)
BEX = 3% beef extract / 0.3M NaCl (pH unadjusted)

In the first of these experiments, oyster tissue was seeded with MS2 and then eluted with 10% tryptose phosphate broth / 0.05 M glycine (pH 9.5), an eluent previously reported for the recovery of viruses from shellfish (Lewis and Metcalf, 1988). Steps 1-6 in Figure 4.2.5 were performed, and the final supernatant was assayed for infectious coliphage MS2. As shown in Table 4.2.2, this procedure and eluent were effective in recovering MS2 from oyster tissue, with 100% of initially seeded virus recovered.

Table 4.2.1 Recovery of infectious of coliphage MS2 from oyster tissue using an elution method

Virus	Oyster sample (grams)	log_{10} PFU MS2 added	log_{10} PFU MS2 recovered (% recovered)
MS2	25.2	6.26	6.32 (100%)

[a]eluent was 10% tryptose phosphate broth / 0.05M glycine (pH 9.5)

This type of experiment was then repeater, with the exception that the elution step was followed by a PEG precipitation step to further reduce the sample volumes as showin in Figure 4.2.5, steps 1-8. Several eluents, including 10% tryptose phosphate broth / 0.05M glycine at both pH 8 and 9.5, 0.5M glycine / 0.1% Tween-80 (pH 8), and 3% beef extract/ 0.3M NaCl (pH unadjusted) were chosen based on previously reported recoveries of rotavirus SA11 from oysters (Lewis and Metcalf,1988). The results of duplicate experiments with both HAV and MS2 seeded in 25 grams of oyster homogenate are presented in Tables 4.2.3, 4.2.4, and Figure 4.2.6. The recoveries of HAV and MS2 were also compared based on elution and PEG precipitation efficiency.

The results of these experiments on MS2 and HAV recovery by elution and PEG precipitation were subjected to statistical analysis. In comparing eluents, there was no statistically significant difference between any of the 4 eluents at eluting HAV (P value = 0.4521; Kruskal-Wallis Test), MS2 (P value is 0.8857; Kruskal-Wallis Test), or both viruses (P value is 0.4521; Kruskal-Wallis Test) from homogenized oysters. Similarly, no statistical significance was found when comparing the 4 eluents based on overall percent recoveries of the method for HAV (P value = 0.2095; Kruskal-Wallis Test), MS2

(P value = 0.5035; Kruskal-Wallis Test), and both viruses (P value = 0.9853; Kruskal-Wallis Test) (Tables 4.2.3 and 4.2.4).

To create more robust set of data for statistical comparisons, recoveries from all 4 eluates were combined. Using this statistical approach, HAV and MS2 were not eluted from oyster homogenate with statistically significantly different results (two tailed P value = 0.2931; Mann-Whitney Test). Sixty percent (± 35%) of HAV and 77% (± 18%) of MS2 was eluted from oyster homogenates and recovered during elution. Unfortunately, only 6.9% (± 5.7%) of HAV in eluates were recovered after PEG precipitation, giving an overall recovery of the method at 4.0% (± 3.2%). Unlike HAV, MS2 was efficiently concentrated from oyster eluates during PEG precipitation giving 54% (±11%) recoveries, and a total overall recovery of 48% (± 11%).

To further test the difference in recoveries from PEG precipitation, a non-parametric ANOVA test was performed showing that the median recovery of MS2 differed significantly from the mean recovery of HAV (two tailed P value = 0.0002; Mann-Whitney Test). As well, significantly more MS2 was recovered than HAV using the direct elution and PEG precipitation method (two tailed P value = 0.0002; Mann-Whitney Test). From these results, it is not clear if these lower recoveries by PEG precipitation compared to initial elution are due to poor HAV precipitation or interference with virus detection by the PEG precipitate itself. As previously indicated, we did not attempt to assay the supernatant following the centrifugation of the PEG material, and so the levels of infectious HAV in the supernatant liquid of the PEG are unknown.

Nevertheless, overall recoveries of infectious HAV were low with a mean of 4.0% (± 3.2%). In the same series of experiments, 0.1 and 10 ml portions of the eluate from unseeded oyster tissue were assayed via the double agar layer or the single agar layer technique for the presence of background levels of both somatic and male-specific coliphages; none were detected in these samples.

Table 4.2.2 Recovery of infectious MS2 from oyster tissue using an elution method followed by polyethylene glycol (PEG) precipitation

Eluant	Average oyster sample (grams)	% MS2 recovered (PFUs)		
		Eluate (St. Dev)	PEG (St. Dev)	Overall (St. dev)
TPB (pH 8)[a]	25.2	69 (±10)	61 (± 3)	50 (± 19)
TPB (pH 9.5)[b]	25.2	76 (± 7)	51 (± 12)	43 (± 0)
0.5M GLY[c]	25.2	75 (± 29)	59 (± 14)	58 (± 13)
3% BEX[d]	25.4	86 (± 26)	43 (± 9)	44 (± 7)

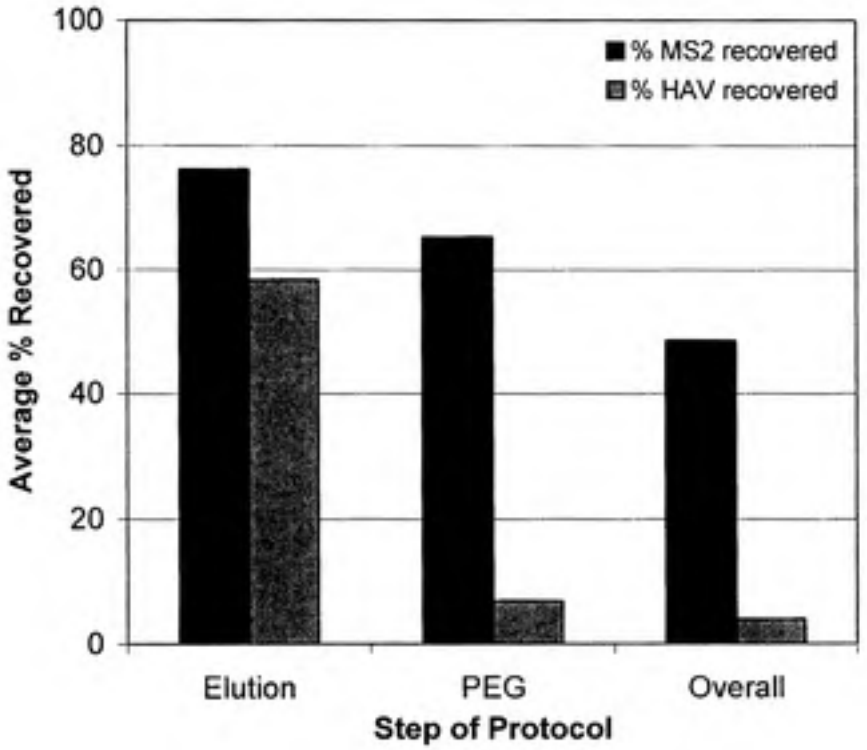
[a]TPB (pH 8) = 10% tryptose phosphate broth / 0.05M glycine (pH 8); [b]TPB (pH 9.5) = 10% tryptose phosphate broth / 0.05M glycine (pH 9.5); [c]0.5M GLY = 0.5M glycine / 0.1% Tween-80 (pH 8); [d]3% BEX = 3% beef extract / 0.3M NaCl (pH unadjusted). [e] MS2 recovered from eluate (refer to Figure 4.4.6- step 6); [f] MS2 recovered from resuspended PEG pellet (refer to Figure 4.4.6- step 8).
[g] PEG recovery is the amount recovered from resuspended PEG pellets divided by the amount recovered from Eluates.
* Overall percent recovered is the total recovered divided by the total input.

Table 4.2.3 Recovery of infectious HAV from oyster tissue using an elution method followed by polyethylene glycol (PEG) precipitation

Eluant	Average oyster sample (grams)	% HAV recovered (PFUs)		
		Eluate (st dev)	PEG (st dev)	Overall (st dev)
TPB (pH 8) [a]	25.2	77 (± 39)	12 (± 12)	7.1 (±4.4)
TPB (pH 9.5) [b]	25.2	73 (± 55)	6.5 (± 0.8)	4.5 (±2.9)
0.5M GLY [c]	25.2	22 (± 0.7)	4.3 (± 2.0)	0.9 (±0.4)
3% BEX [d]	25.4	69 (± 10)	4.7 (± 2.4)	3.4 (±2.1)

[a]TPB (pH 8) = 10% tryptose phosphate broth / 0.05M glycine (pH 8); [b]TPB (pH 9.5) = 10% tryptose phosphate broth / 0.05M glycine (pH 9.5); [c]0.5M GLY = 0.5M glycine / 0.1% Tween-80 (pH 8); [d]3% BEX = 3% beef extract / 0.3M NaCl (pH unadjusted). [e] MS2 recovered from eluate (refer to Figure 4.4.6- step 6); [f] MS2 recovered from resuspended PEG pellet (refer to Figure 4.4.6- step 8).
[g] PEG recovery is the amount recovered from resuspended PEG pellets divided by the amount recovered from Eluates.
* Overall percent recovered is the total recovered divided by the total input.

Figure 4.2.6 Recovery of HAV and MS2 from oyster tissue in duplicated experiments using an elution method followed by polyethylene glycol (PEG) precipitation.



4.2.3 Molecular detection of hepatitis A virus using Conventional OneStep reverse transcriptase polymerase chain reaction (RT-PCR)

For the experiments shown previously, relatively high inputs of virus (*i.e.*, >10000 PFU / gram oyster tissue) were used to ascertain recovery efficiencies of methods and identify any serious deficiencies in specific methods or their key processing steps. In addition, the

methods for elution of viruses from oysters and from the other foods were slightly different. These previous experiments had detected viruses in food samples using a two-step RT-PCR assay, in which viral RNA was either chemically extracted or was physically released from capsids by heating. Viruses also had been detected by infectivity assay.

A OneStep RT-PCR assay was used for the next series of experiments. Compared to the conventional two-step RT-PCR, the OneStep procedure uses smaller overall reaction volumes (25 uL; 2.5 uL of template) compared to the two-step assay (100 uL overall volume; 10 uL of template).

To determine the relationship between HAV cell culture infectivity and RT-PCR signal as amplified by the OneStep RT-PCR assay. Crude virus stocks of HAV were RNA extracted and serially diluted in molecular grade water. The OneStep procedure used a 25 µl reaction volume and 2.5 µl of sample template. A gel-electrophoresed image of one such titration is shown in Figure 4.2.7. There is a faint band in lane 7 that is not of similar size to other amplicons and may be a DNA dimer.

Figure 4.2.7 Titration of HAV HM175 using chemical extraction of RNA followed by OneStep RT-PCR: relationship to infectious units.

Lane 1: 100 bp marker; lane 2: 10^{-3} dilution; lane 3, 10^{-4}; lane 4: 10^{-5}; lane 5: 10^{-6}; lane 6: 10^{-7}. The infectivity titer of this stock of HAV is 7.5×10^{7} PFU/ml; RNA was extracted from 140 ul portions of each dilution and 2.5 ul of RNA extract were used in OneStep RT-PCR reactions. Therefore, the number of RTPCRU in the sample is 10^{7} (1 / 0.14) (1 / 0.025) = 2.9×10^{9} RTPCRU / ml. *192 bp amplicon (Schwab, 1995)*

As shown in Figure 4.2.7, the endpoint of HAV RNA detection for this trial is at the 10^{-7} dilution (the faint band in lane 6), which therefore corresponds to a ratio of 39 RTPCR units:PFU, and is similar to ratios of 25:1 (Casteel 2001) and 23:1 (Schwab, 2000) previously reported. Although, more titrations of stock HAV are required to decrease the uncertainty of the endpoint dilution by OneStep RT-PCR. The same stock of virus that was titrated by the OneStep assay was used in subsequent experiments for HAV recovery from foods followed by detection using RT-PCR.

4.2.4 Sample quality and compatibility with virus detection methods using multiple elution and PEG precipitation steps

To test if there was significant cytotoxicity and/or inhibition of oyster eluates on virus infectivity, which may have caused low recoveries of HAV in resuspended PEG precipitants, unseeded food samples were processed to model steps of the method in Figure 4.2.8 and then processed by OneStep conventional RT-PCR and cell culture infectivity. In the next experiment of this series, 2900 PFU was added to 1 ml of unseeded oyster eluate (*i.e.*, eluate from oysters, steps 1-10 in Figure 4.2.8 without the addition of HAV) or 1 ml of unseeded resuspended PEG material (*i.e.*, steps 1-19). Then, these samples were either diluted in PBS or were further diluted in oyster eluate eluate or resuspended PEG material, and portions were analyzed by infectivity assay. In addition, RNA was extracted from 0.14 ml portions and then assayed via OneStep RT-PCR. The results are shown in Figure 4.2.9 and in Figure 4.2.10.

Figure 4.2.8 Multiple elution and PEG precipitation method for virus recovery and detection

1. Add 175 ml of 0.5 M glycine / 0.1% Tween-80 (pH 8) to tomato sauce, strawberry sauce, or oyster sample seeded with virus
2. Homogenize / blend at high speed for 30 seconds
3. Let blender vessel sit for 10 minutes for foam to dissipate
4. Pour into 500 ml centrifuge bottle; recover remaining foam or liquid in blender vessel with pipette
5. Centrifuge at 2000*xg* for 20 minutes
6. Recover supernatant (supernatant #1)
7. Add 175 ml of fresh eluant to remaining pellet; vortex
8. Centrifuge at 2000xg for 20 minutes
9. Recover supernatant (supernatant #2)
10. Add supernatants #1 and #2 together (approximately 350 ml); adjust to pH 7.2
11. Perform PEG procedure: add 8% PEG and 0.3 M NaCl
12. Transfer into clean 500 ml centrifuge bottle
13. Centrifuge at 4657*xg* for 30 minutes
14. Resuspended PEG pellet in 20 ml of PBS
15. Extract the resuspended PEG material with Vertrel (10 ml)
16. Centrifuge at 2000*xg* for 20 minutes
17. Recover supernatant
18. Perform PEG procedure on supernatant: add 8% PEG and 0.3 M NaCl
19. Resuspend PEG pellet in 2 ml of PBS

As shown in Table 4.2.5, the amount of infectious HAV detected in the oyster eluates and in the PEG precipitates, after dilution in PBS, corresponded both to the amount added initially and to the calculated amounts after dilution. In contrast, the amounts of infectious HAV in samples not diluted in PBS were lower than the calculated amounts. For example, only 120 PFU was recovered from an expected 290 PFU initially inoculated in the oyster eluate, as shown in Table 4.2.5. HAV inoculated into "clean" samples (oyster eluate diluted in PBS) and assayed by cell culture were recovered in quantities the same as that of the inoculum, while HAV inoculated into "dirty" samples (eluates diluted in oyster eluates) had dramatically lower plaque counts in cell culture infectivity assays. Similar cell culture results were found for PEG precipitated oyster samples diluted in PBS instead of PEG eluates and assayed for HAV infectivity. This demonstrates that there was interference of the processed oyster sample material with detection by infectivity. Inhibition was also seen when HAV RNA was amplified by OneStep RT-PCR and visualized by gel electrophoresis. As shown in Table 4.2.5 and in Figure 4.2.10, PFU equivalents of HAV RNA were not detected in samples containing < 290 PFU/ml and < 2 PFU per 2.5 ul of template, although the detection limit of the OneStep RT-PCR assay in deionized water is 0.05 PFU per 2.5 ul of template with 25 ul total reaction volumes. HAV RNA inoculated in oyster extracts or PEG extracts, and diluted in PBS were detected as positive when PFU equivalents HAV RNA were in quantities 1-2 logs above the detection limit the assay in deionized water (Table 4.2.5, Figure 4.2.8). This experiment demonstrates that neither simple direct elution nor subsequent PEG concentration efficiently remove enzymatic inhibitors from oyster tissue, although sample quality is acceptable for sensitive detection with cell culture infectivity.

Table 4.2.4 Detection of infectious HAV (and HAV RNA; see Fig. 4.2.9) in unseeded eluates and unseeded PEG material after specific stages of sample processing

Oyster sample	Dilution	Total PFU HAV inoculated in 1 ml of sample	Total PFU HAV detected in sample	PFU found in 0.14 ml	Estimated PFU per RT-PCR Reaction	Positive by Gel Electrophoresis (see below)	Lane in gel (see below)
Eluates: dilutions made in PBS	undilute	2900	TNTCc	406	20.3	Yes	2
	1:10	290	272	38	2.03	Yes	3
	1:100	29	35	4.9	0.23	No	4
	1:1000	2.9	3.5	0.49	0.023	No	5
Eluates: dilutions made in mock eluate	undilute	2900	TNTCc	406	20.3	Yes	6
	1:10	290	120	16.8	2.03	No	7
	1:100	29	8	1.1	0.23	No	8
	1:1000	2.9	<1PFU	< 1 PFU	0.023	No	9
PEGb: dilutions made in PBS	undilute	2900	–d	406	20.3	Yes	10
	1:10	290	272	38.1	2.03	Yes	11
	1:100	29	29	4.1	0.23	No	12
	1:1000	2.9	5	0.7	0.023	No	13
PEGb: dilutions made in mock PEG	undilute	2900	–d	406	20.3	Yes	14
	1:10	290	–d	40.6	2.03	Yes	15
	1:100	29	–d	4.06	0.23	No	16
	1:1000	2.9	–d	0.406	0.023	No	17

[a]Eluate = TPB (pH 8) = 10% tryptose phosphate broth / 0.05M glycine (pH 8); [b]PEG = PEG pellet (from TPB (pH 8)), resuspended in 20 mL of PBS; [c]slight cytotoxicity; plaques visible; [d]– significant cytotoxicity; plaques barely visible, therefore, no data obtainable

* the detection limit of cell culture infectivity assay is 1 PFU per 200 ul sample

Figure 4.2.9 OneStep RT-PCR of RNA chemically extracted from HAV samples that had been seeded into oyster eluate and then precipitated with PEG (seeTable 4.2.5 for sample descriptions)

Lane 1: 100 bp marker; lanes 2-17 (see Table 4.4.4 for description); lane 18: negative control *192 bp amplicon (Schwab, 1995)*

4.2.5 Recovery of HAV from complex foods using multiple elution and PEG steps

Because the "simple" direct elution method (Figure 4.2.6) with additional PEG concentration (Figure 4.2.8) gave poor sample quality, the method was expanded to allow for steps that not only recover viruses, but also increase sample quality by reducing inhibitors that interfere with viral RNA detection by RT-PCR (Figure 4.2.10). In the next series of experiments attempts were made to recover and detect low input levels of HAV (*i.e.*, $\leq$1000 PFU per sample) seeded in the semi-solid / liquid food matrices, including oyster tissue, tomato sauce, and blended strawberries. A glycine buffer (0.5 M Glycine/0.1% Tween-80 (pH 8.0)) was chosen as the eluent, because statistical tests showed no significant difference (P value = 0.4521; Kruskal-Wallis Test),) between the glycine buffer and 3 other published buffers (Lewis and Metcalf, 1988) for the ability to recover HAV from oysters. Also, the glycine buffer is hypothesized to contribute less to inhibition of RT-PCR than other buffers in this study, including 3% beef extract which

contains a chaotropic agent sodium nitrate known to inhibit RT-PCR (Sheih et al., 1997). The recovery procedure used is shown in Figure 4.2.10. In the following experiments detection of HAV was for viral RNA amplified by OneStep RT-PCR.

Figure 4.2.10 Multiple elution and PEG method

1. Add 175 ml of 0.5 M glycine / 0.1% Tween-80 (pH 8) to 25 grams of tomato sauce, strawberry sauce, or oyster sample seeded with virus
2. Homogenize / blend at high speed for 30 seconds
3. Let blender vessel sit for 10 minutes for foam to dissipate
4. Pour into 500 ml centrifuge bottle; recover remaining foam or liquid in blender vessel with pipette
5. Centrifuge at 2000*xg* for 20 minutes
6. Recover supernatant (supernatant #1)
7. Add 175 ml of fresh eluant to remaining pellet; vortex
8. Centrifuge at 2000*xg* for 20 minutes
9. Recover supernatant (supernatant #2)
10. Add supernatants #1 and #2 together (approximately 350 ml); adjust to pH 7.2
11. Perform PEG procedure: add 8% PEG and 0.3 M NaCl
12. Transfer into clean 500 ml centrifuge bottle
13. Centrifuge at 4657*xg* for 30 minutes
14. Resuspended PEG pellet in 20 ml of PBS
15. Extract the resuspended PEG material with Vertrel (10 ml)
16. Centrifuge at 2000*xg* for 20 minutes
17. Recover supernatant
18. Perform PEG procedure on supernatant: add 8% PEG and 0.3 M NaCl
19. Resuspend PEG pellet in 2 ml of PBS
20. Vertrel extraction (5 ml)
21. Recover supernatant
22. Chemically extract RNA; dilute extracts 1:10 to 1:100 in PCR grade water
23. Assay undilute and diluted RNA extracts using OneStep RT-PCR assay

These recovery and detection methods were performed on duplicate oyster samples and on one sample each of tomato and strawberry sauce. The results of experiments with seeding levels of HAV at 100 PFU per sample and 1000 PFU per sample are shown in Figure 4.4.11 and Figure 4.4.12, respectively. Expected or theoretical values for cell culture inoculums and OneStep RT-PCR are given in Table 4.4.5.

As shown in Figure 4.4.11, HAV RNA was not detected in any of the samples seeded initially with 100 PFU (390 RTPCRU). However, portions of the resuspended PEG

sample (step 19, Figure 4.4.10) were assayed for HAV by infectivity assay. Infectious HAV was detected in one of the oyster samples and in the strawberry sauce sample, at a total recovery value of 5 PFU (data not shown). In contrast, infectious HAV was not detected in the other oyster sample, but a total of 25 PFU was recovered and detected from the tomato sauce sample. This efficiency of recovery of HAV after PEG precipitation of eluates is low and is similar to the values reported in Table 4.2.4 (about 40-100%).

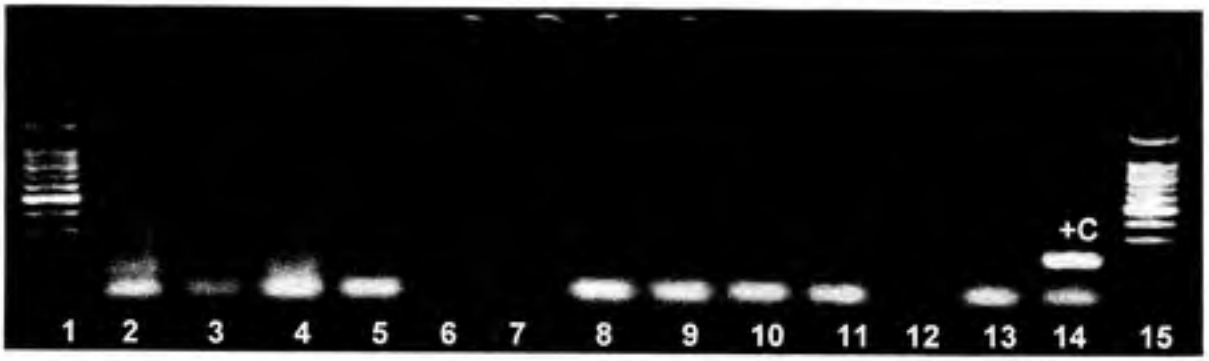
To determine if a higher level of HAV could be recovered and detected in food samples using the recovery procedure shown in Figure 4.4.10, oyster, tomato sauce, and blended strawberry samples were seeded with 1000 PFU of HAV. Like the last experiment, HAV RNA was not detected in any of the samples (Figure 4.4.11). No attempt was made to detect infectious HAV in the samples from this experiment.

Table 4.2.5 Companion to Figures 4.4.12 and 4.4.13 giving expected levels of HAV in cell culture inoculums and OneStep RT-PCR reactions, based on food and initial inoculum.

Food	HAV Input (PFU)	Expected PFU in 200 ul	HAV RNA dilution factor	Expected PFU in 2.5 ul	Lane in gel
Oyster 1	1000	50	undilute	3.5	2
			1:10	0.35	3
Oyster 2	1000	50	undilute	3.5	4
			1:10	0.35	5
Tomato Sauce	1000	50	undilute	3.5	8
			1:10	0.35	9
Strawberries	1000	50	undilute	3.5	10
			1:10	0.35	11
Oyster 1	100	50	undilute	3.5	2
			1:10	0.35	3
Oyster 2	100	50	undilute	3.5	4
			1:10	0.35	5
Tomato Sauce	100	50	undilute	3.5	8
			1:10	0.35	9

Strawberries	100	50	undilute	3.5	10
			1:10	0.35	11

Figure 4.2.11 OneStep RT-PCR of HAV recovered from tomato sauce, strawberry sauce, and oyster samples (food samples seeded with 100 PFU or 3900 RTPCRU)



Lane 1: 100 bp marker; lane 2: undiluted HAV RNA from oyster sample #1; lane 3: 1:10 dilution of sample in lane 2; lane 4: oyster b RNA undlute; lane 5: 1:10; lanes 6 and 7: empty; lane 8: tomato RNA und; lane 9:1:10; lane 10:strawberry RNA undiluted; lane 11: strawberry RNA 1:10; lane 12: empty; lane 13: negative control; lane 14: positive control; lane 15: 100 bp marker. Sample sizes were as follows: tomato sauce (31 grams); strawberry sauce (28 grams); oysters #1 and #2 (9 grams each). This corresponds to a seed level of 3.2 PFU/g, 3.6 PFU/g, and 11 PFU/g for TS, SS, and oysters, respectively. *192 bp amplicon (Schwab, 1995)*

Figure 4.2.12 OneStep RT-PCR of HAV recovered from tomato sauce, strawberry sauce, and oyster samples (food samples seeded with 1000 PFU or 39000 RTPCRU)

Lane 1: 100 bp marker; lane 2: undiluted HAV RNA from oyster sample #1; lane 3: 1:10 dilution of sample in lane 2; lane 4: oyster b RNA undilute; lane 5: 1:10; lanes 6 and 7: empty; lane 8: tomato RNA und; lane 9:1:10; lane 10:strawberry RNA undiluted; lane 11: strawberry RNA 1:10; lane 12: empty; lane 13: negative control; lane 14: positive control; lane 15: 100 bp marker. Sample sizes were as follows: tomato sauce (31 grams); strawberry sauce (28 grams); oyster #1 (6 grams) and oyster #2 (14 grams). This corresponds to a seed level of 32 PFU/g, 36 PFU/g, 167 PFU/g , and 71 PFU/g for TS, SS, and oysters 1 and 2, respectively. *192 bp amplicon (Schwab, 1995)*

In summary, the experiments using alkaline elution methods to recover HAV from foods suggest that the elution procedure was somewhat effective in recovering viruses. Viruses were eluted from oysters homogenates using previously reported eluents (Lewis and Metcalf, 1988), and 60 (± 35%) of initially seeded HAV and 77% (± 18%) of initially seeded MS2 were recovered. Although MS2 was efficiently concentrated by PEG, the data show low recoveries of HAV after PEG concentration 6.9% (± 5.7%), which could mean that either the PEG procedure was either not efficient in precipitating HAV in food eluates, or that there was significant cytotoxicity and/or inhibition to RT-PCR, even after chemical RNA extraction. Experiments to determine sample quality showed no reduction in observed plaque counts as compared to expected plaque counts for oyster tissue samples diluted in PBS, but significant cytotoxicity for oyster samples diluted in other oyster extracts not containing virus. Oyster extracts were detected by OneStep RT-PCR as positive only when PFU equivalents HAV RNA in were in quantities 1-2 logs above the detection limit the assay. Even after methodological modification to reduce inhibitors, such as organic solvent extraction with Vertrel XF®, HAV nucleic acids were not detected when initially seeded at 1,000 PFU HAV or 100 PFU HAV in oysters, tomato sauce, and strawberries. These low inoculum experiments were done at 1-2 logs above the detection limit of the OneStep RT-PCR assay. The use of elution methods to recover low levels of HAV in foods, and detect HAV genomic RNA using RT-PCR was not as successful as initially anticipated. Further work towards developing methods to recover HAV and MS2 from oysters, produce, and sauces must focus on (i) efficiently recovering and concentrating viruses from foods; (ii) increasing the sensitivity of

detection of molecular methods; and (iii) removing inhibitors that interfere with sensitive detection.

4.3 Virus Nucleic Acid Amplification and Detection

4.3.1 Molecular detection of hepatitis A virus using realtime RT-PCR, and nested RT-PCR

From experience gained during development of alkaline elution methods, it was determined that not only improved recovery methods were required, but more sensitive and robust molecular detection methods should also be incorporated in the study. Two candidate molecular detection methods, realtime RT-PCR and nested RT-PCR, were investigated for their ability to detect low levels of HAV genomic RNA in both deionized water and sample matrices. The following section describes the results of titrations of HAV RNA towards developing sensitive molecular detection methods.

Realtime RT-PCR was used to detect and quantify HAV RNA in samples. One common method for analysis and quantification of unknown samples is by comparison to a standard curve, as explained in the Materials and Methods section. In realtime RT-PCR, standard curves were derived from serial tenfold dilutions of a sample of known concentration and validated by calculating the reaction efficiency and slope coefficient as explained in the Methods and Materials section. Standard curves generated for the detection of HAV RNA in a stock virus sample and analyzed using software included with the Cepheid Smart Cycler (Sunnyvale, CA) are show below in Table 4.3.1). Dividing the actual concentration (PFU) of virus recovered in a sample by the expected

concentration of virus present as assayed by cell culture infectivity (assumed to be 100%) gives the percent detection of HAV RNA for a sample. Detection of HAV was calculated for different experimental conditions of probe label, probe concentration and sample dilution and results are shown below in Table 4.3.1.

Table 4.3.1 Data from quantitative realtime RT-PCR standard curves for hepatitis A virus

Date of Assay	Probe Label	Probe Concentration	Tenfold Dilutions	Slope Coefficient	Reaction Efficiency
5-Aug-03	5' ROX - BHQ 3'	0.1 uM	4	0.99	0.98
14-Aug-03	**5' ROX - BHQ 3'**	**0.1 uM**	**5**	**1.00**	**0.98**
21-Oct-03	5' ROX - BHQ 3'	0.2 uM	4	0.98	0.85
26-Nov-03	5' ROX - BHQ 3'	0.2 uM	4	0.99	1.24
26-Nov-03	5' ROX - BHQ 3'	0.2 uM	5	0.99	1.10
11-Nov-03	**5' ROX - BHQ 3'**	**0.2 uM**	**4**	**0.96**	**0.95**
12-Apr-04	**5' ROX - BHQ 3'**	**0.2 uM**	**4**	**0.99**	**0.98**
5-Jun-04	5' ROX - BHQ 3'	0.2 uM	4	1.00	0.85
23-Jun-04	5' FAM - BHQ 3'	0.2 uM	5	0.98	0.90
18-Jun-04	**5' FAM - BHQ 3'**	**0.2 uM**	**6**	**1.00**	**1.03**
Expected			5 or more	1.00	1.00

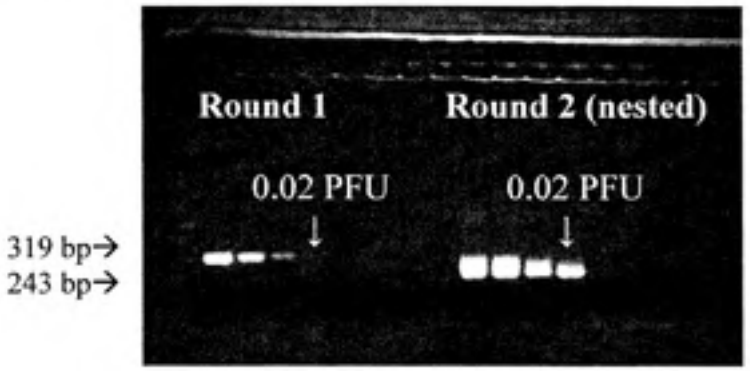
* FAM and ROX are fluorescent labeled dyes situated on the 5' prime end of the probe, and BHQ stands for Black Hole Quencher situated on the 3' end of the probe.

As shown in Table 4.3.1, 11 standard curves were generated using 2 different probes, a FAM-labeled probe and a ROX-labeled probe. Characteristics of a perfect standard curve are: reaction efficiency = 1, slope coefficient = 1, and 5 or more serial tenfold sample dilutions. All trials in Table 4.3.1 use 4 or more serial tenfold dilutions to generate standard curves. Reaction efficiencies ranged from 0.85 to 1.03, and slope coefficients ranged from 0.96 to 1.00. Cepheid software applies the cycle threshold (Ct) values of unknown samples and uses it to extrapolate the concentration of virus in unknown samples based on a selected standard curve. Trials highlighted in Table 4.3.1 are standard curves chosen for comparison with curves generated by analysis of unknown samples. The basis for their selection is that these trials all have reaction efficiencies

(between 0.95 and 1.03) more closely approximating perfect reaction efficiency than other standard curves. Standard curves were generated for comparison to curves generated from unknown samples processed with different probe sequences, concentrations, and stocks of HAV used at various stages of the research. For example, the standard curve from August 14, 2003 uses a ROX probe at a 0.1 μM concentration and was generated for initial experiments before the probe concentration was optimized to 0.2 μM. Later work included a FAM labeled probe, because newer RT-PCR kit chemistries necessitated such a change.

We hypothesized that realtime RT-PCR may be more sensitive to food-based inhibitors than other molecular methods, such as nested or conventional RT-PCR. Realtime RT-PCR may also facilitate the visualization of food-based inhibition, because the assay uses as a measure the rate of exponential increase of PCR product amplification, rather than measuring the final quantity amplified product as in conventional PCR. In this way realtime RT-PCR indicates the efficiency of amplification as a function of the rate of the reaction. To compare realtime RT-PCR to another sensitive molecular detection method, a nested RT-PCR system was developed using published primers (Hutin et al., 1999) and a one-tube, one-step kit (Qiagen, Valencia, CA). The detection limit of the nested assay using stock HAV extracted with a Viral RNA Extraction Mini kit (Qiagen, Valencia, CA), amplified from a sample volume of 5 μl in an initial reaction volume of 25 μl and visualized by gel electrophoresis was ~ 0.02 PFU HAV in one experiment (Figure 4.3.1).

Figure 4.3.1 Titration of stock Hepatitis A virus by Nested RT-PCR.



Lane 1- ladder, Round 1 bands = 20 PFU, 2 PFU, 0.2 PFU, 0.02 PFU
Round 2 (nested) bands = 20 PFU, 2 PFU, 0.2 PFU, 0.02 PFU

Assuming the final visible band in the nested assay contains 1 RT-PCR unit, a ratio of 50 RTPCR:PFU was present, which is similar to previously reported ratios of 39 (Section 4.2.3), 25:1 (Casteel 2001) and 23:1 (Schwab et al,, 2000). This stock of virus was used in subsequent experiments for HAV recovery from foods followed by molecular detection and/or cell culture infectivity. Titration of the same stock of HAV by realtime RT-PCR consistently detected HAV at a higher amount (1 to 3 logs more) than nested RT-PCR detected during one trial. Although, more titration of HAV by nested RT-PCR are required to further ground this claim.

4.4 Acid-adsorption Elution Methods

4.4.1 Development of acid-adsorption elution methods to recover viruses from fruit and vegetable (non-oyster) food matrices

After developing alternatives to conventional RT-PCR for the sensitive detection of HAV nucleic acids, the study began investigating alternative methods for recovering and concentrating viruses from foods. Because of the early observation that the acidic pH of tomato and strawberry sauce (*i.e.*, pH ~ 4) resulted in adsorption of virions to the sauce material, we hypothesized that an acid-adsorption elution technique, which has been previously employed for the recovery of viruses from shellfish tissue, would perhaps also work on recovering viruses from other kinds of food samples. As well, oyster tissue processed by acid-adsorption elution methods retains fewer RT-PCR inhibitors than elution methods (Shieh et al., 1999). Possibly, acid-adsorption elution methods could reduce more inhibitors from produce and sauces than elution methods. Initial tests were conducted using coliphage MS2 and HAV in tomato and strawberry sauce. The procedure shown in Figure 4.4.1 (up to step 16) was used, and samples were assayed using the double agar layer technique as described in Materials and Methods.

Figure 4.4.1 Acid-adsorption of viruses to tomato sauce and blended strawberries

1. Add 7 volumes (vol:vol) chilled and sterile deionized water to tomato sauce and strawberry sample (30 grams)
2. Homogenize in Waring Blender on high for 2 min. Pour into 250 mL centrifuge bottle
3. Adjust pH to 4.8-5.0
4. Shake (horizontally) at 100 RPM for 15 min
5. Centrifuge at 2000 *xg* for 20 min at 4°C
6. Retain liquid supernatant; assay by cell culture infectivity and RT-PCR for HAV and by double agar layer for MS2

These experiments are intended to show that virus particles can be (1) effectively adsorbed to the food-matrix; and (2) viruses bound to food particulates would pellet upon centrifugation, leaving no viruses in the supernatant. After centrifugation, the supernatant was assayed for viruses as a proxy for the amount of virus present in the pellet. That is, the total input of virus minus the virus present in the supernatant to be discarded would estimate the quantity of viruses present in the pellet. Data in Table 4.4.1 indicates that, when processed by the protocol in Figure 4.4.1, tomato sauce pellets contain less HAV than strawberry sauce pellets. Subtracting the virus present in the supernatant from the total virus input shows 67.1% (± 6.2) and 56.9% (± 12.8) of the total HAV inoculum is present in tomato sauce pellets (Figure 4.4.1- step 5), as detected by cell culture infectivity and realtime RT-PCR, respectively (Table 4.4.1). Strawberry pellets from the same analysis protocol and processing step contain 89.2% (± 5.37) and 87.6% (± 5.6) of the total HAV inoculum, as detected by cell culture infectivity and

realtime RT-PCR, respectively (Table 4.4.1). Data from another virus, MS2, run in the same experiment gives a similar trend for these food samples (Table 4.4.2). MS2 loss in discarded supernatant (Figure 4.4.1- step 5) was low, with 7.0% (± 1.0%) recovered from discarded tomato sauce supernatant, and 0.9% (± 0.3%) recovered from discarded strawberry supernatant. Subtracting virus loss from total virus input gives 99.1% (± 0.3%) MS2 left in strawberry pellets, and 93% (± 1.0%) MS2 left in tomato sauce pellets (Figure 4.4.2). For HAV and MS2, the tomato sauce matrix is somewhat less efficient at retaining acid-adsorbed virus particles than a strawberry sauce matrix (Figure 4.4.2).

Table 4.4.1 HAV recovered from a discarded supernatant during acid-adsorption elution method

Matrix	HAV	% Recovered (st dev)*	
	PFU input	**Cell Culture**	**Realtime RT-PCR**
Tomato Sauce	4.24×10^5	32.9 (± 6.2)	43.1 (± 12.8)
Strawberry Sauce	4.24×10^5	10.8 (± 5.37)	12.6 (± 5.6)

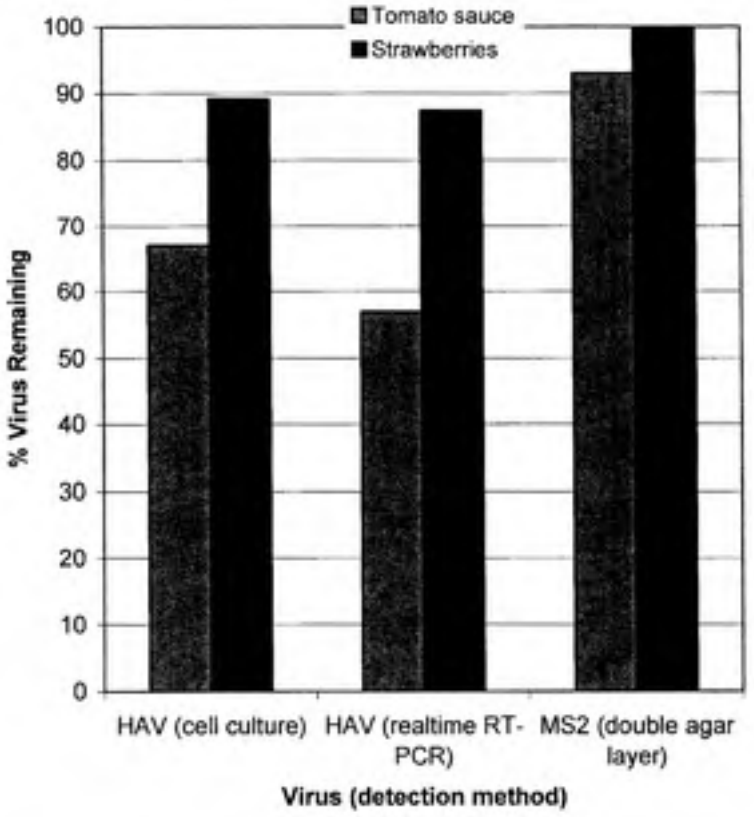
* n = 2

Table 4.4.2 MS2 recovered from the discarded supernatant of the acid-adsorption elution method

Matrix	**MS2**	**% Recovered (st dev)***
	PFU input	**Double Agar Layer**
Tomato Sauce	3.91×10^7	7.0 (± 1.0)
Strawberry Sauce	3.91×10^7	0.9 (± 0.3)

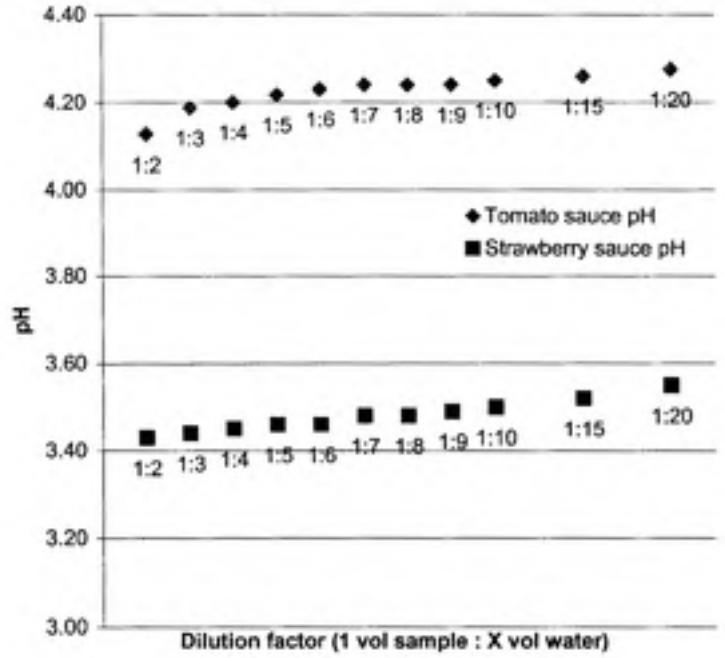
* n = 2

Figure 4.4.2 Efficiency of HAV and MS2 Acid Adsorption to Foods



To better understand the effects of sample conditions in the crucial acid-adsorption steps, the effects of both pH and conductivity were measured as a function of the dilution of sauces in deionized water (Figures 4.4.3 and 4.4.4). Tomato sauce and strawberry sauce were diluted 1:2, 1:3, 1:4 etc., till 1:20 and samples were measured incrementally for pH and conductivity. Figure 4.4.3 shows that as samples become more dilute, the conductivity decreases— dramatically in the case of tomato sauce and less so for strawberry sauce. Optimal conductivity for the adsorption of viruses to oyster tissue is 2000 μS/cm, while optimum conductivities for virus to other food matrices have not been determined. Figure 4.4.4 shows the effects of dilution on ambient food pH are small. Through dilution, tomato sauce pH increases from an undiluted pH of ~4.1 to a 1:20

Figure 4.4.4. Effect of sample dilution on pH of tomato sauce and strawberries



The results of these experiments indicated that food conductivity decreased greatly and pH increased a little by dilution with water. If sample conductivity and pH are important factors influencing virus adsorption, the large decrease in the eluent conductivity of tomato sauce with dilution could result changes in adsorption efficiency, along with a possible effect from the minor change in sample pH.

In previous studies on virus recovery by elution and acid-adsorption elution, 1:4 to 1:10 vol:vol dilutions of sample in eluent are typical. In the acid-adsorption studies described above, 1:7 sample dilutions are used. In this study the effects of sample pH and conductivity on virus adsorption and elution were not systematically investigated.

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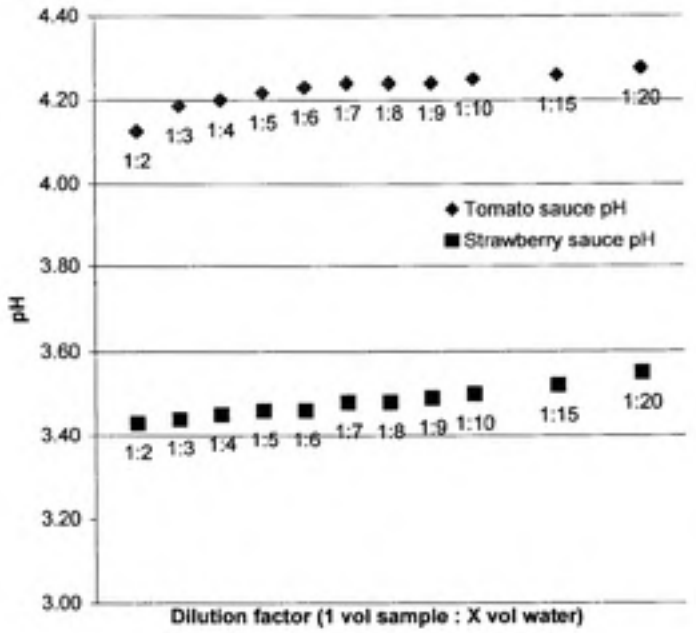

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Therefore, it is possible that optimization of these conditions would increase virus recoveries over those reported in Tables 4.4.3 and 4.4.5.

Additional experiments were then designed and carried out to optimize or further improve the previously observed poor adsorption of virus to tomato sauce. One experiment investigated how 2 different pH levels (ambient food pH and pH 4.8-5), and three centrifugation speeds (2,000*xg*, 4,000*xg*, and 6,000*xg*) affected the ability of HAV to adsorb to foods and be effectively pelleted. A total of 4.24×10^5 PFU of HAV was inoculated into 30 grams of tomato sauce and processed using the protocol from Figure 4.4.1. Table 4.4.3 presents the results of realtime RT-PCR to detect the percent of viruses recovered from the discarded supernatant (Figure 4.4.1, step 6) as a proxy for the virus-containing pellet. The average HAV recovered from the discarded supernatant ranged from 8.8% to 37.1%, meaning that the retained pellet contained a range of 91.2% to 62.9% of the total initial HAV inoculum.

Using a non-parametric ANOVA test, the means of samples processed by different combinations of pH and centrifugation do not differ more than expected by chance alone (P value = 0.4349; Kruskal-Wallis Test). In other words, no combination of pH and centrifugation speed is significantly different than another. When comparing centrifugation speeds, there is no reason to conclude that the mean recoveries of HAV differ when one of three centrifugations (2,000*xg*, 4,000*xg*, and 6,000*xg*) was applied (P value = 0.2601; Kruskal-Wallis Test). Furthermore, the percent of HAV recovered from samples adjusted to pH 4.8-5 was not significantly different (two tailed P value = 0.5887;

Mann-Whitney Test) from the percent HAV recovered from samples adjusted to pH ~4.2. Therefore, HAV recoveries from tomato sauce at ambient pH are probably the same as for samples adjusted to pH 4.8-5. Furthermore, significant time savings could be realized by maintaining ambient tomato sauce pH instead of adjusting to pH 4.5-5, thereby simplifying the method.

Samples not seeded with virus also were included in these experiments. After processing these unseeded samples for virus recovery, they were then inoculated with HAV RNA, and processed with HAV-seeded samples by realtime RT-PCR to determine if there was sample-related inhibition. As shown in Table 4.4.4, of the 21.2 PFUs of HAV inoculum added (as enumerated by cell culture infectivity), realtime RT-PCR detected 20.1 ± 3.6 PFUs, giving a 94.8% recovery. Hence, the processed sample was relatively free of inhibitors of realtime RT-PCR amplification.

Next, tomato sauce samples with three conductivities (2,000 µS/cm, 4,000 µS/cm, and 6,000 µS/cm) and three pH levels (pH 4, 5, and 6) were compared to determine the optimal conditions for adsorption of HAV to tomato sauce. Specifically, HAV was inoculated in 30 grams of tomato sauce and adjusted to one of three conductivities resulting in a concentration of ~7,000 PFU HAV/ml. All samples were processed using the protocol from Figure 4.4.1 The results in Table 4.4.5 show that HAV in the discarded supernatant ranged from 5.2% to 32.4%, meaning a range of 94.8% to 67.6% of HAV was present in the retained the pellet. Median recoveries of HAV were not significantly greater with any one conductivity (2,000 µS/cm, 4,000 µS/cm, or 6,000 µS/cm) than provided by chance alone (P value = 0.6286; Kruskal-Wallis Test). Similarly, sample pH

did not have a statistically significant effect on the median percent recovery of HAV from tomato sauce (P value = 0.5429; Kruskal-Wallis Test).

As previously, tomato sauce samples free of virus were generated, then inoculated with HAV RNA, and processed with virus-seeded samples by realtime RT-PCR to determine if there was inhibition of the amplification reaction. Table 4.4.6 shows that the 31.15 PFU inoculum (as enumerated by cell culture infectivity) was detected by realtime RT-PCR as 30.7 ± 4.4 PFUs, giving a 98.6% recovery.

Table 4.4.3 HAV recovered from water wash eluates adjusted to 2 different pHs and centrifuged 3 different speeds.

Tomato Sauce	HAV Input	pH	Centrifugation	Realtime RT-PCR **				
Sample	PFU			Ct value	PFU per reaction	% Recovered	Average % Recovered	St. Dev of % Recovered
A	4.24×10^5	pH 4.8-5	2,000 xg	38.92	0.416	1.0	11.8	15.3
B	"			37.74	0.957	22.6		
A	"	pH 4.8-5	4,000 xg	39.39	0.299	7.1	8.8	2.4
B	"			38.83	0.443	10.4		
A	"	pH 4.8-5	6,000 xg	39.9	0.208	4.9	13.2	11.7
B	"			37.81	0.911	21.5		
A	4.24×10^5	pH ~4.2 *	2,000 xg	40.25	0.162	3.8	9.2	7.7
B	"			38.36	0.621	14.6		
A	"	pH ~4.2	4,000 xg	38.46	0.557	13.1	8.9	6.0
B	"			39.97	0.198	4.7		
A	"	pH ~4.2	6,000 xg	37.54	1.107	26.1	37.1	15.6
B	"			36.67	2.042	48.2		
Negative Control	0	pH 4.8-5	2,000 xg	0	--	--	--	--

* pH 4.2 is ambient pH for tomato sauce mixed in 100 ml water; ** samples assayed at 1:10 dilution in RNase free deionized water

Table 4.4.4 Tomato sauce inhibition to the detection of HAV by realtime RT-PCR

Tomato Sauce	HAV Input	Realtime RT-PCR *				
	PFU	Ct value	PFU	Average PFU	St. Dev of Average PFU	Average % Recovered
Sample Negative Control + HAV	21.2	36.64	22.64	20.1	3.6	94.8
Sample Negative Control + HAV	21.2	37.04	17.557			
Sample Negative Control	0	0	0	--	--	--

Table 4.4.5 HAV recovered from water wash eluates adjusted to 3 different pHs and 3 different conductivities.

Tomato Sauce	HAV Input	Conductivity	pH	Realtime RT-PCR*				
Sample	[concentration]			Ct value	PFU per reaction	% Recovered	Average recovered	St dev
A	2.18×10^6 PFU [6922 PFU/ml]	6,000 uS/cm	4	36.53	2.246	32.4	17.0	13.9
			5	37.81	0.909	13.1		
			6	39.07	0.376	5.4		
B	1.03×10^6 PFU [6922 PFU/ml]	4,000 uS/cm	4	37.19	1.415	20.4	14.8	8.4
			5	39.12	0.36	5.2		
			6	37.31	1.301	18.8		
C	6.23×10^5 PFU [6922PFU/ml]	2,000 uS/cm	4	37.37	1.247	18.0	22.9	6.1
			5	37.16	1.442	20.8		
			6	36.66	2.058	29.7		
Negative Control	0	2,000 uS/cm	5	0	0	--	--	--

* samples assayed at 1:10 dilution in RNase free deionized water

Table 4.4.6 Tomato sauce inhibition to the detection of HAV by realtime RT-PCR

Tomato Sauce	HAV Input	Realtime RT-PCR *				
Sample	(PFU)	Ct value	PFU	Average PFU	St. Dev of Average PFU	Average % Recovered
Sample Negative Control + HAV	31.15	32.7	33.783	30.7	4.4	98.6
Sample Negative Control + HAV	31.15	32.99	27.572			
Sample Negative Control	0	0	0	--	--	--

* samples assayed at 1:10 dilution in RNase free deionized water

4.4.2 Recovery of hepatitis A virus during PEG, RNA extraction and RNA precipitation

The recovery of HAV during PEG precipitation based on viruses eluted from the resuspended PEG pellet, RNA extraction, and RNA precipitation was investigated. Positive controls (HAV inoculated into PBS) were used to compare the effects of centrifugation temperature on recovery and to compare isopropanol to ethanol as a precipitating agent. Previously, it was assumed that short (5 min) room temperature centrifugation was adequate for the pelleting of RNA during ethanol precipitation, but after several negative results this assumption was questioned. With ethanol precipitation it was found that chilling the microcentrifuge at -20°C before the spin and centrifuging at 4°C, resulted in increased virus recovery over room temperature centrifugation (data not shown). A comparison of isopropanol to ethanol found the two comparable at concentrating and precipitating virus RNA, which is consistent with the findings of others. Though comparable, ethanol was not chosen as a precipitating agent because 50% less isopropanol by volume is required for nucleic acid precipitation, thus minimizing the total volume to be centrifuged for RNA recovery (Sambrook *et al.*, 1989).

Other ways to improve virus recoveries from positive control samples were attempted by increasing the centrifugal forces and times for PEG centrifugation. In this experiment, 1 x 10^5 PFU HAV was inoculated into PBS (pH 7.5), then PEG precipitated overnight at 4 °C (8% PEG; 0.3 M NaCl), and centrifuged using the centrifugal forces and times shown. The PEG pellet was extracted with GuSCN buffer, RNA extracted in large volumes (~4 ml), and the RNA was eluted from Midi columns using 200 µl of TE buffer plus 10 U of RNasin. The recovered RNA was precipitated using isopropanol, resuspended in 20 µl of

TE buffer, and 5 µl of RNA was analyzed by realtime RT-PCR (Jothikumar et al., 2004). As shown in Table 4.4.7 below, doubling the centrifugation time increases HAV recovery in one trial, but more experimental replicates are needed to justify this claim. Sample RNA was serially tenfold diluted in molecular grade water, and assayed as 1:10 and 1:100 dilutions because inhibition was present in undiluted samples—hypothesized to be from residual alcohol in nucleic acid precipitated pellets or from inhibitors in the crude virus stock.

Table 4.4.7 Effect of spin time on recovery of HAV from PEG

Sample	PEG pellet centrifugation	Virus input PFU HAV	Dilution	qRT-PCR analysis Ct value	% Recovered
PBS	15,000*xg* for 15 min	1×10^5	1:10	33.53	12%
			1:100	36.48	12%
PBS	15,000*xg* for 30 min	1×10^5	1:10	31.33	48%
			1:100	33.82	96%

5 log10 PFU HAV added to sample; therefore, if one assumes 100% recovery , the theoretical amount of HAV present in 5 uL of sample taken for RT-PCR is: ((100,000 PFU / 0.02 mL) (0.005 mL) = 25,000 PFU; percent recovery = PFU recovered / 25,000 PFU (100).

An experiment was done to investigated the limit of detection of HAV in PBS (pH 7.5) samples processed by polyethylene glycol precipitation, centrifugation for 30 minutes at 15,000*xg* and 4°C, direct GuSCN lysis of the PEG pellet, Midi column extraction, isopropanol precipitated to 20 ul, and 5 ul of 10-fold or 100-fold dilutions of alcohol concentrated RNA extracts were used in a realtime RT-PCR assay (Table 4.4.8) (Jothikumar et al., 2004). As shown in Table 4.4.8, inoculums of <1,000 PFU HAV could not be detected by realtime RT-PCR. However, the detection limit of a titration of stock HAV used as a standard curve in realtime RT-PCR (Table 4.3.1) was 10 PFU, and the theoretical detection limit is an initial inoculum of 400 PFU HAV. In three samples,

HAV inoculums of ≥ 1,000 PFU were detected in amounts ranging from 48% to 120% of the initial inoculum. The experiment has two limitations: 1) the detection limit was rather high (10 PFU); 2) sample inhibition interfered with detection in undilute samples. Perhaps lower levels of HAV RNA detection may be possible through nested RT-PCR or Southern Blot analysis of amplified DNA. The same experiment as in Table 4.4.8 was repeated later in tomato sauce and strawberry matrices (Tables 4.4.9 and 4.4.10).

Table 4.4.8 Detection limit of HAV inoculated in PBS and PEG precipitated

Sample	HAV Inoculum (PFU)	PEG Centrifugation	Real-time RT-PCR analysis Dilution	Ct value	% Recovered
PBS	1×10^5 *	15,000 *xg* 30 min at 4°C	1:10	31.33	48%
			1:100	33.82	96%
	1×10^4	"	1:10	33.87	93%
			1:100	37.09	120%
	1×10^3	"	1:10	38.08	64%
			1:100	0	<<10 PFU**
	1×10^2	"	1:10	0	<<10 PFU
			1:100	0	<<10 PFU
	1×10^1		1:10	0	<<10 PFU
			1:100	0	<<10 PFU

*5 log10 PFU HAV added to sample; therefore, if one assumes 100% recovery, the theoretical amount of HAV present in 5 uL of sample taken for RT-PCR is: ((100,000 PFU / 0.02 mL) (0.005 mL) = 25,000 PFU; percent recovery = PFU recovered / 25,000 PFU (100); for 4 log10 input, use 2,500 PFU, etc.
**<<10 PFU. The last detectable dilution of the realtime RT-PCR assay (used as a standard curve) was 10 PFU HAV; giving a theoretical limit of detection of 400 PFUs of initial inoculum.

4.4.3 Limit of detection of hepatitis A virus in food eluates using realtime RT-PCR

Two determinants of successful virus recovery in food samples are (1) the compatibility of samples with a detection method; and (2) the sensitivity or lower detection limit of a method. Further experiments tested the compatibility of food samples processed by acid-

adsorption elution, concentration, and purification steps in Figure 4.4.5, for detection of HAV in realtime RT-PCR.

Figure 4.4.1 Acid-adsorption elution method for the recovery of viruses from complex food samples

1. Add 105 ml of chilled, sterile, distilled water to tomato sauce, strawberry sauce, or oyster sample in 250 ml centrifuge bottle
2. For oysters only: adjust conductivity to <2,000 µS/cm; adjust pH to 4.8-5.0
3. Shake (horizontally) all samples at 100 RPM for 15 minutes
4. Centrifuge at 2000xg for 20 minutes
5. Discard water supernatant
6. Resuspend pellet in 105 mL of 0.05 M glycine / 0.14 M NaCl (pH 7.5)
7. Add 1:2 :: Vertrel : sample; vortex 2 min.
8. Centrifuge at 5000xg for 30 minutes
9. Save eluate #1
10. Resuspend pellet in 105 mL of 0.5M threonine / 0.14 M NaCl (pH 7.5)
11. Centrifuge at 4657xg for 20 minutes
12. Save eluate #2
13. Combine eluate #1 and eluate #2 (approximately 210 ml)
14. Add 8% PEG and 0.3 M NaCl: stir to solution; store overnight 4°C
15. Centrifuge 15,000 xg for 30 minutes
16. Discard supernatant
17. Resuspend PEG pellet in 5 mL GuSCN
18. Recover RNA through Midi spin column
19. Isopropanol precipitate RNA
22. Resuspend precipitate in 20 uL of 10 uM Tris-1mM EDTA (TE; pH 8.0) + 10 U RNasin
23. Aassay 5 uL RNA extract using realtime RT-PCR

Sample inhibition was investigated for representative foods, specifically, strawberries, tomato sauce, dissected oyster stomachs, and whole oysters. In this single replicate experiment, ~4,000 PFU equivalents of HAV RNA was added (in step 22 of the method in Figure 4.4.5) to each serial tenfold diluted RNA extracted food sample. It was hypothesized that food eluates lacking inhibitors to RT-PCR should provide similar and high recoveries at each dilution, with recoveries at the 1:10 dilution being similar to those at 1:100 and 1:1000 dilutions, because the same PFU equivalents of HAV RNA were added to each dilution. As shown in Table 4.4.8, strawberry puree and tomato sauce processed by the method in Figure 4.4.5 were not appreciably inhibitory to HAV RNA

detection by realtime RT-PCR. Virus detection at the 1:10 dilution of extracted RNA was not inhibitory to RT-PCR, nor were the 100- and 1000-fold dilutions. Tomato sauce also was not greatly inhibitory to realtime RT-PCR detection, as percent recoveries were high at all sample dilutions tested. However, Ct values and percent recoveries did increase with increasing sample dilution, indicating some inhibition of realtime RT-PCR by this type of sample. Processed samples of both dissected and whole oysters were inhibitory to realtime RT-PCR detection, as Ct values and percent recoveries were lowest in samples diluted the least (10-fold) and highest in samples diluted the most (1000-fold).

Table 4.4.1. Inhibition from complex food eluates to the detection of HAV in realtime RT-PCR

Sample	Food (g)		Realtime RT-PCR analysis		
	Total	Assayed in 1 reaction	Dilution	Ct value	% recovery
Strawberry	15	0.26	1:10	30.49	203%
		0.026	1:100	30.46	207%
		0.0026	1:1000	30.58	192%
Tomato Sauce	15	0.26	1:10	30.6	189%
		0.026	1:100	29.89	298%
		0.0026	1:1000	29.62	354%
Dissected Oyster *	16	0.28	1:10	41.1	0%
		0.028	1:100	34.62	14%
		0.0028	1:1000	30.15	253%
Whole Oyster	17	0.3	1:10	33.77	25%
		0.03	1:100	31.34	118%
		0.003	1:1000	30.54	196%

~4,000 (3.6 log10) PFU HAV added to sample; therefore, if one assumes 100% recovery, the theoretical amount of HAV present in 5 µl of sample taken for RT-PCR is: ((3.6 log 10 / 0.02 ml) x (0.005 ml) = 1000 PFU; percent recovery = PFU recovered / 1000 PFU (100)

Each of the food samples in the experiments described above are 15- to 17-gram sub-sample of larger pre-homogenized sample pools, such as a homogenized jar of tomato

sauce, or a homogenized box of frozen strawberries. Furthermore, the need for sample dilution to overcome remaining RT-PCR inhibition from the sample reduces the sensitivity of the method and decreases the sample size that can be analyzed.

Oyster samples were the most inhibitort to HAV RNA detection by realtime RT-PCR, with only 25 percent of the HAV RNA inoculum detectable at the 1:10 dilution in whole oyster samples. At the 1:100 sample dilution over 100% of the initial HAV RNA inoculum was detectable. Dissected oysters required 10-fold greater dilution than whole oysters to give relatively inhibition-free detection of HAV RNA. It is presumed that dissected oysters provide a larger effective sample size because most viruses accumulate in this tissue as compared to other shellfish tissues. Although one dissected oyster weighs about one tenth as much as a whole oyster, when comparing equal weights of dissected and whole oysters there is less inhibition with whole oysters. This observation suggests that the levels of inhibitory materials are high in the digestive gland tissues, compared to the levels in other tissues of the oyster. Interestingly, the PEG pellet from a whole oyster sample is twice as small as the PEG pellet from a comparable dissected oyster sample. The reasons for this are uncertain but it is possible that whole oysters, containing more muscle tissue, resulted in pelled solids that are easier to remove during clarification steps (Figure 28, step 4) than the solids from the digestive tissue.

Further work to remove or overcome food-based inhibitors examined the ability of a GuSCN lysis buffer, (whose recipe is listed in methods and materials) to digest various foods. Weighed food samples, specifically oyster stomachs, tomato slices, ground beef,

strawberries, and wheat crackers, were placed in 1:2, 1:1, or 2:1 volume ratios of GuSCN lysis buffer and observed after 15 minutes, 30 minutes, 60 minutes and overnight. As indicated by the observation recorded in Table 4.4.9, GuSCN was effective at digesting fats, sugars, and to some extent proteins from foods. Higher concentrations of GuSCN resulted in faster digestion of foods. Blended strawberries were completely dissolved/digested, while tomato slices, ground beef and oyster stomachs were partially to half dissolved/digested after 24 hours. Interestingly, strawberry seeds and plant ligneous tissue were not digested by GuSCN lysis buffer even after 24 hours. Wheat crackers seemed unaffected by GuSCN, probably because the sample matrix was low in moisture content so that the digestion processes could not proceed. GuSCN did not dissolve/digest dissected oyster stomachs as rapidly as other investigators report oyster stomach digestion with 8 commercially available proteases (Legeay, Caudrelier et al. 2000).

Ultimately, direct GuSCN digestion of foods did not appear to be an acceptable replacement of acid-adsorption elution or elution as means to recover viruses from internally contaminated foods because the lysis buffer cannot completely digest many of the samples of interest. However, some investigators have used GuSCN washes to recover HAV and noroviruses from surface contaminated foods like lunch meats (Schwab et al., 2000). In that study, GuSCN washes were preferable to surface elution of viruses with phosphate buffered saline (Schwab et al., 2000). In the current study, the preferred approach to virus recovery from intact foods was to wash with an elution medium that recovers virions and then purify and concentrate the recovered virions in the eluate for subsequent RNA extraction and RT-PCR detection.

Table 4.4.2 Digestion of foods with GuSCN lysis buffer: a qualitative analysis

Sample	Mass (grams or ul)	Ratio of sample to GuSNC lysis buffer (mass/volume)	Digestion of sample at time intervals: 15 min	30 min	60 min	overnight
Oyster stomach	0.21 g	2:1	no effect---------->			
	0.23 g	1:1	contents increasingly digested---------->			
	0.28 g	1:2	contents increasingly digested---------->			Stomach contents intact
Tomato slices	0.25 g	2:1	no effect---------->		partially dissolved	
	0.22 g	1:1	no effect---------->		partially dissolved	
	0.23 g	1:2	partially digested			partially digested
Ground Beef	0.21 g	2:1	no effect---------->			
	0.2 g	1:1	no effect---------->			
	0.19 g	1:2	partially dissolved---------->			50 % still intact
Strawberry sauce	40 ul	2:1	Viscous---------->			
	40 ul	1:1	runny---------->			
	40 ul	1:2	Liquid with seeds present---------->			Liquid with seeds present
Wheat crackers	0.09 g	2:1	No effect; Crackers absorbed the lysis buffer			
	0.075 g	1:1	No effect; Crackers absorbed the lysis buffer			
	0.087 g	1:2	No effect; Crackers absorbed the lysis buffer			

To further examine the effects of sample inhibition on detection of HAV from RNA extracted foods, the recovery of HAV was studied by spiking a range of HAV levels ($1x10^3$ PFU to $1x10^5$ PFU) into foods at step 13 of the method in Figure 4.4.5. This is the point in the procedure where viruses have been eluted from the food solids and are then going to be precipitated with PEG. After PEG precipitation, RNA extraction and concentration and isopropanol precipitation, the resulting HAV RNA was serial tenfold diluted in molecular grade water and assayed by realtime RT-PCR. Undiluted samples from tomato sauce and strawberries were not assayed, because enzymatic inhibitors greatly reduced detectablity of HAV nucleic acids. The results in Table 4.4.10 show that all tomato sauce and blended strawberry samples inoculated with $\geq 1 \times 10^4$ PFU HAV are detected by realtime RT-PCR. With a 1×10^4 PFU inoculum, RNA extracted strawberry samples diluted as much as 1:1000 in deionized water are detected by realtime RT-PCR. The lowest detectable dilution giving positive RT-PCR signals for tomato sauce was a 1:10 dilution in deionized water. More replicates should be performed to better characterize the differences in recovery between tomato sauce and blended strawberries. In general, when tomato sauce and strawberry samples are extensively concentrated by PEG precipitation, 4 ml RNA extraction, and isopropanol nucleic acid precipitation, the large inputs of HAV are either lost during processing steps or excessive concentration inhibits accurate sample detection and quantification.

Table 4.4.3 Recovery and detection of HAV from strawberry sauce eluates using realtime RT-PCR

Sample	Food (grams)		Virus input		Realtime RT-PCR analysis		
	Total	Assayed in 1 reaction	PFU HAV	Dilution	Theoretical PFU assayed	Ct value	% Recovered
Strawberry Sauce	15	0.26	1×10^5 *	1:10	2,500	32.67	20%
		0.026		1:100	250	35.23	39%
		0.0026		1:1000	25	37.83	72%
Strawberry Sauce	15	0.26	1×10^4	1:10	250	35.48	33%
		0.026		1:100	25	39.02	36%
		0.0026		1:1000	2.5	39.55	250%
Strawberry Sauce	15	2.6	1×10^3	Undilute	250	ND	<<10 PFU **
		0.26		1:10	25	ND	<<10 PFU

** [(actual PFU recovered / theoretical PFU present) * 100 = the % recovered]

** <<10 PFU. Based on the last detectable dilution of the realtime RT-PCR, the theoretical limit of detection was 400 PFUs of initial inoculum.

ND = Not Detected

Table 4.4.4 Recovery and detection of HAV from tomato sauce eluates using realtime RT-PCR

Sample	Food (g)		Virus input		Realtime RT-PCR analysis		
	Total	Assayed in 1 reaction	PFU HAV	Dilution	Theoretical PFU assayed	Ct value	% Recovered
Tomato Sauce	15	0.26	1×10^5 *	1:10	2,500	33.44	12%
		0.026		1:100	250	37.1	12%
		0.0026		1:1000	25	41.04 **	<<10 PFU ***
Tomato Sauce	15	0.26	1×10^4	1:10	250	39.07	3%
		0.026		1:100	25	ND	<<10 PFU
		0.0026		1:1000	2.5	ND	<<10 PFU
Tomato Sauce	15	2.6	1×10^3	Undiluted	250	ND	<<10 PFU
		0.26		1:10	25	ND	<<10 PFU

[(actual PFU recovered / theoretical PFU present) * 100 = the % recovered]

** this sample was assigned a Ct value by extrapolating of a fitted line, but Ct values >40 generally not used in realtime RT-PCR.

*** <<10 PFU. Based on the last detectable dilution of the realtime RT-PCR, the theoretical limit of detection was 400 PFUs of initial inoculum.

ND = Not Detected

The results in Table 4.4.8 above indicated that strawberry sauce and tomato sauce were compatible with detection by realtime RT-PCR dilutions of the final RNA, ranging from 1:10 for strawberry sauce to 1:1000 for dissected oyster tissue. The results in Tables 4.4.10 and 4.4.11 indicated low virus recovery efficiency even high HAV input levels. A similar recovery experiment was performed on dissected oysters with HAV inputs of 1 x 10^5 and 1 x 10^4 PFU with no positive signals detected by realtime RT-PCR. While tomato sauce and strawberry sauce can be successfully processed and assayed for HAV by acid-adsorption elution and realtime RT-PCR when contamination levels are high and sample extracts are diluted, dissected oysters and whole oysters apparently are more difficult matrices from which to recover virus and reduce RT-PCR inhibitors, as no viruses were detectable by the same processing and assay methods.

4.4.4 Characterizing food-based inhibitors by nested RT-PCR, realtime RT-PCR, and spectrophotometry

The results for HAV RNA detection by realtime PCR of strawberry and tomato sauce samples processed by adsorption-elution-PEG precipitation and RNA extraction indicated remaining inhibitors and relatively poor virus detection or recovery for tomato sauce. Therefore, experiments were done to determine if lower detection levels of input virus could be achieved using nested RT-PCR, which potentially is less affected by inhibitors.

Both alkaline amino acid elution and acid-adsorption elution have been previously shown to be effective for recovering viruses from various food samples. However, following several concentration and purification steps, it was shown in this study that detection of low-levels of viruses in eluates from complex food samples using conventional RT-PCR

was inhibited, even following PEG, fluorocarbon extraction, and/or RNA chemical extraction steps. To understand where the inhibition problems occurred in these steps and to improve virus recovery per step, several experiments were performed in which HAV was added to samples (such as in the experiments shown in Table 4.4.8) taken from different stages in the overall protocol. Samples were processed by realtime RT-PCR and nested RT-PCR to compare the two RNA detection methods. Other samples were analyzed by visible spectrophotometry at 230 nm to determine the levels of plant-based inhibitors, based on the absorbance of polysaccharides.

From results in the previous section, it was hypothesized that realtime RT-PCR may be more sensitive to food-based inhibitors than other molecular methods, such as nested or conventional RT-PCR. Realtime RT-PCR may also be better suited to quantitatively examine the effects of food-based inhibition, because the assay uses as a measure the exponential rate of amplification of PCR product, rather than measuring the final quantity of product amplified— as in conventional PCR. However, the realtime RT-PCR method appears to be quite sensitive to RT-PCR inhibitors remaining in processed samples, thereby limiting the ability to detect small quantities of viral RNA in large quantities of food.

To better understand food-based inhibition on virus detection using an acid-adsorption elution method (Figure 4.4.6, short form and Figure 4.4.7), a single trial experiment was devised where RNA extracts of food that contained no added viruses were inoculated with known amounts of HAV RNA after step 18 of the method in Figure 4.4.7. To quantify the effect of sample dilution on HAV RNA detection, 200 PFU of HAV RNA

was inoculated in undiluted and serial tenfold dilutions of tomato sauce and blended strawberries. To quantify sample inhibition of a decreasing amount of HAV RNA with increasing sample dilution, HAV RNA was added to food samples and the whole RNA extract of the sample concentrate was serial tenfold diluted and analyzed by RT-PCR.

Figure 4.4.2 Overview of short and long acid-adsorption elution methods

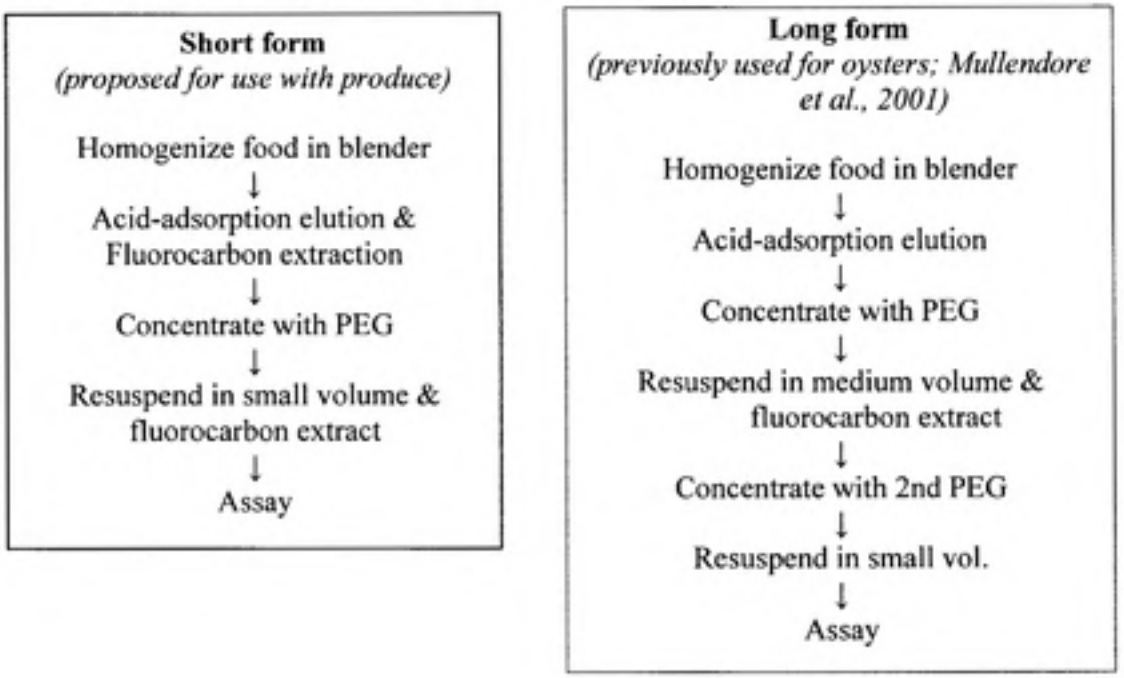


Figure 4.4.3 Short acid-adsorption elution method to recover HAV from produce

1. Add 7 volumes (vol:vol) water to tomato sauce and strawberry sample (30 grams)
2. Homogenize in Waring Blender on high for 2 min. Pour into 250 mL centrifuge bottle
3. Adjust pH to 4.8-5.0
4. Shake (horizontally) at 100 RPM for 15 min
5. Centrifuge at 2000 *xg* for 20 min; Discard water supernatant
6. Resuspend pellet in 7 vol (vol:vol) 0.05 M glycine / 0.14 M NaCl (pH 7.5) or 0.75 M glycine/0.14 M NaCl (pH 7.5)
7. Add 0.5 vol (vol:vol) Chloroform and shake 2 min
8. Centrifuge at 5000 *xg* for 30 min;
    a. recover supernatant (eluate # 1)
9. Resuspend interface in 50mL of 0.5M threonine / 0.14 M NaCl (pH 7.5)
10. Shake 2 min, centrifuge at 4657*xg* for 20 minutes; recover supernatant (eluate # 2)
11. Combine eluate #1 and eluate #2
12. Add 8% PEG and 0.3 M NaCl: stir to solution; store overnight 4°C
13. Centrifuge 6,000*xg* for 30 minutes
14. Resuspend pellet in3-5 mL PBS (pH 7.5)
15. Add 0.5 vol Chloroform; vortex; centrifuge at 3000 *xg* for 15 min
16. Recover supernatant; off-gas in petri dish
17. Split the sample: 1 ml for infectivity;
    a. the rest for molecular detection
18. RNA extract whole sample (3-5 ml) using Qiagen 4 ml Midi column
19. Isopropanol precipitate RNA extracts with NH_4OAc; Resuspend in 10 ul TE (pH 8.0)
20. Assay 2.5 ul RNA by qRT-PCR and nested RT-PCR

Table 4.4.12 shows the inhibition of HAV RNA detection still present in a realtime RT-PCR reaction, as a function of the grams of food in that reaction. A positive control consisting of 200 PFU of HAV in deionized water was detected as 232 PFU by standard curve analysis, giving 116% recovered. A positive control titration (20 PFU, 2 PFU, etc.) in deionized water would likely give similar results as for 200 PFU, based on the high reaction efficiencies calculated for realtime RT-PCR standard curves shown previously (Table 4.3.1). With tomato sauce and strawberry mock samples, % percent recovery increases as the sample becomes more dilute, and as the virus is diluted in parallel with sample dilutions. In both foods as undiluted samples (7.5 grams of food per reaction), considerable inhibition of HAV RNA detection by realtime RT-PCR, with 1% recovery of HAV from tomato sauce mock samples, and 8% recovery in strawberry sauce. With

the 1:10 dilution (0.75 grams of food per reaction) some inhibition of HAV RNA detection by realtime RT-PCR was present in tomato samples, although there was 56 and 71% recovery. In blended strawberry samples inhibition of HAV RNA detection by realtime RT-PCR was minimal in the reactions, with 87% and 113% recovery.

Table 4.4.5 Realtime RT-PCR detection of hepatitis A virus in foods by short acid-adsorption elution method

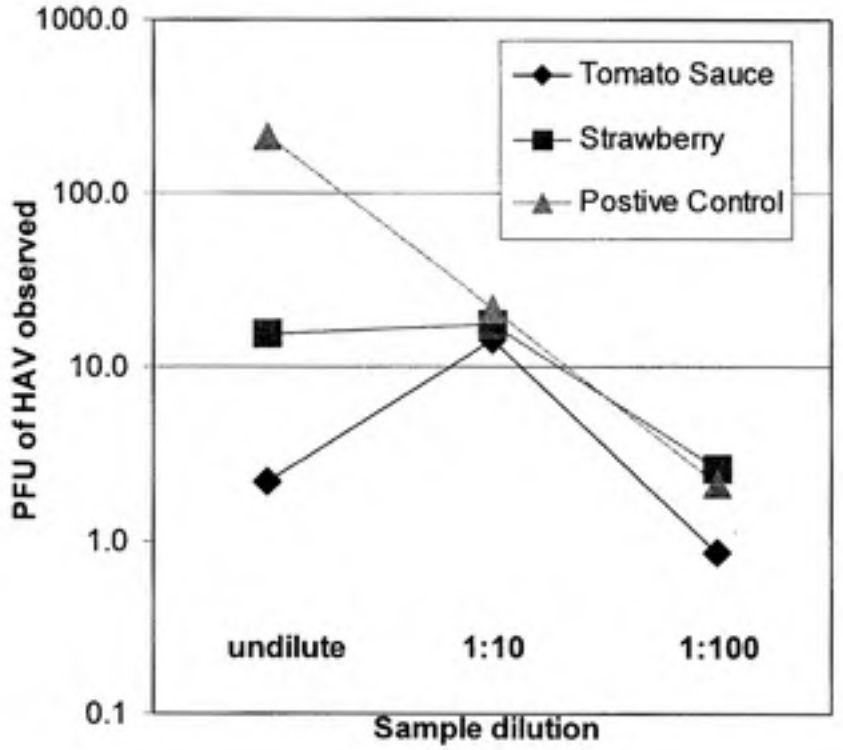
Sample	Food		q-RT-PCR			
	Total	1 RT-PCR reaction	Dilution	PFU input* of HAV	Ct value	% recovery
Tomato Sauce	30 g	7.5 g	Undlilute	200	37.57	1%
		0.75 g	1:10	200	32.33	56%
		0.075 g	1:100	200	31.6	97%
		7.5 g	Undlilute	200	37.57	1%
		0.75 g	1:10	20	35.08	71%
		0.075 g	1:100	2	38.83	42%
Strawberry	30g	7.5 g	Undlilute	200	34.95	8%
		0.75 g	1:10	200	31.4	113%
		0.075 g	1:100	200	30.75	184%
		7.5 g	Undlilute	200	34.95	8%
		0.75 g	1:10	20	34.8	87%
		0.075 g	1:100	2	37.34	129%
Positive Control				200	31.36	116%

*HAV RNA was spiked into 10 ul of mock foods, or into 10-fold dilutions of mock foods– at final step of method (Figure 4.3.6, step 19)

The results from Table 4.4.12 are shown graphically in Figure 4.4.8. This graph represents part of Table 4.4.12, with declining virus input (200, 20 and 2 PFU) with greater dilution (undilute, 1:10, 1:00 dilutions). The graph present the equivalent PFU of HAV RNA detected in realtime RT-PCR reactions as a function of sample dilution. The positive control gives the desired quantitative relationship of decreasing HAV RNA detection in a dilution series, and has a straight line with a negative slope for a reaction in

which no RT-PCR inhibition is present. Undiluted food samples (7.5 grams of food per reaction), especially tomato sauce, are greatly affected by food-based inhibitors that decrease HAV RNA detection. The food-based inhibitors are less problematic as food samples become more dilute. The effects of sample dilution, not inhibition, are seen in 1:10 and 1:100 diluted food samples.

Figure 4.4.4 Effect of sample dilution on detection of HAV RNA by realtime RT-PCR

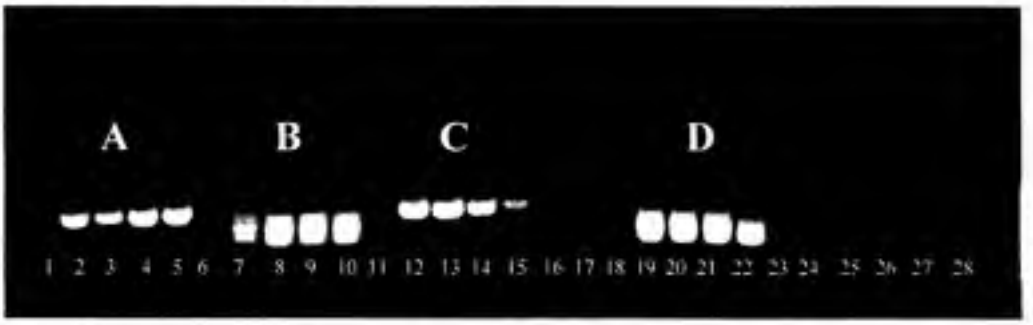


Parallel experiments on the possible role of food-based inhibitors as determined by analysis of serially 10-fold diluted food extract samples for HAV RNA detection also were done using Nested RT-PCR. Nested RT-PCR samples were processed and analyzed with the same virus stock, the same RNA extraction, and the same dilution series as realtime RT-PCR samples. As above for the results of realtime RT-PCR, the

effects of sample dilution on HAV RNA detection by nested RT-PCR were seen when 200 PFU of HAV RNA was inoculated in undiluted and serial tenfold dilutions of tomato sauce and strawberry (Figures 4.4.9 and 4.4.10 below). To examine possible inhibition of HAV RNA amplification of nested RT-PCR with virus dilutions, HAV RNA was added to undiluted food samples and the whole sample was serial tenfold diluted to the endpoint of virus detection. Samples are shown below in Figures 4.4.9 and 4.4.10. Only qualitative or semi-quantitative data can be obtained from examination of amplicon band intensity in electrophoresed and ethidium-bromide stained agarose gels amplified from samples by the nested RT-PCR assay. This is in contrast to the more quantitative nature of realtime RT-PCR in which the rate of increase of a chemical fluorescent probe in the amplicons is visualized. The amplicons in 1st and 2nd round (nested) amplification of undiluted food samples (7.5 grams of food per reaction) inoculated with HAV look as intense as bands from 1:10 and 1:100 dilutions (0.75 and 0.075 grams of food per reaction, respectively), suggesting little inhibition of HAV RNA detection. However, it is difficult to estimate the amount of PCR product in an observed DNA band. However, there appears to be little to no inhibition in the undiluted samples that were processed and analyzed (7.5 grams of food per reaction). From Figure 4.4.9 it appears that the titration of HAV in food extracts tomato sauce samples gave 1 log less sensitivity to detection than the titration of HAV in water (Figure 4.3.1), although one trial is not enough give an accurate interpretation. Strawberry samples inoculated with PFU equivalents of HAV RNA could be diluted to 0.002 PFU as shown in Figure 4.4.10, but this must be human error because RT-PCR cannot detect less than 1 particle, and a ratio of 50 RTPCR units

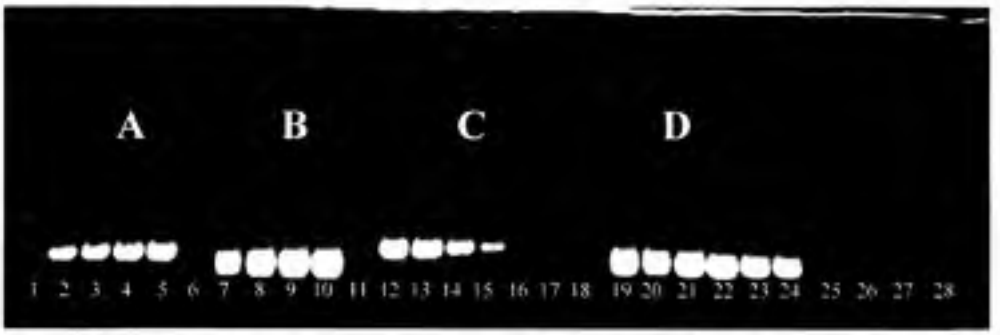
to PFU was determined for nested RT-PCR in water (Figure 4.3.1). It is more likely that, if repeated, the detection limit of HAV RNA in strawberries is 0.02 PFU per reaction.

Figure 4.4.5 Effect of food sample dilution on nested RT-PCR detection of HAV RNA in tomato sauce extracts.



Lanes 1 and 28 contain a 100 kb DNA ladder. Lanes 2 (*first round 319 bp*) and 7 (*nested round 243 bp*) are HAV positive controls. Lanes 6,11, and 18 are blank. Lanes 25,26, and 27 are negative controls with water instead of HAV template during1st round, 1st to 2nd round, and 2nd round replication, respectively. Groups labeled "A" are first round products and groups labeled "B" are second round (nested) products from the same experiment. For example, lane 3 is undilute first round product, with lane 8 as its corresponding nested band. Lanes 3-5 and lanes 8-10 are tenfold dilution series of tomato sauce extracts with 200 PFU equivilants of HAV RNA inoculated in each sample. Groups labeled "C" are first round products and groups labeled "D" are second round (nested) products from the same experiment. For example, lane 12 is undilute first round product, with lane 19 as its corresponding nested band. Lanes 12-17 and lanes 19-24 are from a tenfold dilution series of tomato sauce extracts with 200 PFU equivalents of HAV RNA inoculated in the undilute sample (lane 12). The undilute sample was serial tenfold diluted to the -5 dilution.

Figure 4.4.6 Effect of food sample dilution on nested RT-PCR detection of HAV RNA in strawberry extracts.



Lanes 1 and 28 contain a 100 kb DNA ladder. Lanes 2 (*first round 319 bp*) and 7 (*nested round 243 bp*) are HAV positive controls. Lanes 6,11, and 18 are blank. Lanes 25,26, and 27 are negative controls with water instead of HAV template during1st round, 1st to 2nd round, and 2nd round replication, respectively. Groups labeled "A" are first round products and groups labeled "B" are second round (nested) products from the same experiment. For example, lane 3 is undilute first round product, with lane 8 as its corresponding nested band. Lanes 3-5 and lanes 8-10 are a tenfold dilution series of strawberry extracts with 200 PFU equivilants of HAV RNA inoculated in each sample. Groups labeled "C" are first round products and groups labeled "D" are second round (nested) products from the same experiment. For

example, lane 12 is undilute first round product, with lane 19 as its corresponding nested band. Lanes 12-17 and lanes 19-24 are a dilution series of strawberry extracts with 200 PFU equivalents of HAV RNA inoculated in the undilute sample (lane 12). The undilute sample was serial tenfold diluted to the -5 dilution.

To further explore possible plant-based RT-PCR inhibitors as found in tomato sauce and strawberries, the approaches used in plant virology were explored. Plant virologists attempt to recover viruses from ligneous tissue, leaves, flowers, roots, and fruit by freezing in liquid nitrogen then grinding in a mortar and pestle before the virus is extracted and further purified. Plant virologists use a spectrophotometer to gage the quality of an RNA extracted sample before molecular detection. Analysis by spectrophotometer includes measuring the absorbance at 230 nanometers (nm), a wavelength used to detect the presence polysaccharides, a common RT-PCR inhibitor, and the absorbance of RNA/DNA at 260 nm. Polysaccharides are removed from RNA extracts by the addition of lithium chloride ($LiCl_2$), a monovalent cation, during alcohol precipitation. Adding lithium chloride during alcohol precipitation allows DNA, nucleotides, and plant polysaccharides to stay in solution, while precipitating RNA.

A comparison was made between lithium chloride and other commonly used monovalent cation salts for their ability to purify RNA samples. In one experiment, 450 µl of tomato sauce extract was isopropanol precipitated in the presence of 4 monovalent cations (Table 4.4.13). Samples were kept for 30 minutes at –20 °C, then centrifuged at 14,000 rpm for 15 min, washed with 70% EtOH, respun for 5 minutes, and the resulting pellet was resuspended in TE buffer (pH 8). Dilutions of the resuspended pellet were assayed in a Spectrophotometer at an absorbance of 230 nm; the wavelength at which plant polysaccharides best absorb light. Samples were compared to a negative control that had

no cation treatment. Lithium chloride performed as well as sodium acetate at removing plant polysaccharides, and slightly better than ammonium acetate or sodium chloride. All treatments performed better than the negative control.

Table 4.4.6 Monovalent cations in alcohol nucleic acid precipitation and their ability to solubalize plant polysaccharides in tomato sauce

Monovalent Cation	Molarity (M)		Absorbance
	Stock	Final	of sample at 230 nm
$LiCl_2$	8	0.8	0.800
NH_4Oac	5	0.5	0.960
NaOAc	3	0.3	0.808
NaCl	5	0.2	1.002
Negative Control	0	0	1.128

Although lithium chloride performs best at reducing plant polysaccharides and is recommended in the plant virology literature, it does a poor job of precipitating small quantities of RNA. It is recommended that at least 0.2-0.4 ug/ul of RNA be present to ensure high recoveries of RNA by lithium chloride precipitation. Lithium chloride is recommended in plant virology because sample quality, not sample size or quantity of nucleic acids is the primary concern for plant virologists. Plant viruses grow to very high concentration in infected cells and tissues, so the amount of target viral nucleic acid is usually much higher than encountered in environmental virology for enteric viruses in water and food. Environmental virologists almost never have the recommended concentration of viral RNA to effectively perform lithium chloride precipitation.

Table 4.4.14 gives the mass of HAV RNA in micrograms (μg) based on the absorbance (A260) of 10^5 PFU of HAV RNA. As seen in Table 4.4.14, the concentration of RNA in a typical sample is far lower than the recommended amount of RNA for lithium chloride

precipitation. An alternative monovalent cation, ammonium acetate, is used in this study because it works relatively well at purifying RNA and allows for the precipitation of small quantities of viral RNA.

Table 4.4.7 Mass of HAV RNA based on Spectrophotometric analysis

HAV (PFU)	Mass of HAV RNA (μg)	250 ul Sample Concentration (μg/μl)
105	36	0.144
104	3.6	0.0144
103	0.36	0.00144
102	0.036	0.000144
10	0.0036	0.0000144
1	0.00036	0.00000144

4.4.5 Recovery efficiency of viruses in complex foods using acid-adsorption elution methods

Further experiments were undertaken to recover coliphage MS2 from tomato sauce and blended strawberries (also referred to as strawberry sauce) using an acid-adsorption elution method. The procedure shown in Figure 4.4.11 (up to step 16) was used, and samples were assayed using the double agar layer plaque technique as described in Materials and Methods.

Figure 4.4.7 Acid-adsorption elution method for the recovery of viruses from complex food samples

1. Add 105 mL of chilled, sterile, distilled water to tomato sauce, strawberry sauce, or oyster sample in 500 mL centrifuge bottle

2. For oysters only: adjust conductivity to <2,000 uS/cm; adjust pH to 4.8-5.0

3. Shake (horizontally) all samples at 100 RPM for 15 minutes

4. Centrifuge at 2000*xg* for 20 minutes

5. Discard water supernatant

6. Resuspend pellet in 105 mL of 0.05 M glycine / 0.14 M NaCl (pH 7.5)

7. Centrifuge at 5000*xg* for 30 minutes

8. Save eluate #1

9. Resuspend pellet in 105 mL of 0.5M threonine / 0.14 M NaCl (pH 7.5)

10. Centrifuge at 4657*xg* for 20 minutes

11. Save eluate #2

12. Combine eluate #1 and eluate #2 (approximately 210 mL)

13. Add 8% PEG and 0.3 M NaCl: stir to solution; store overnight 4°C

14. Centrifuge 4657*xg* for 30 minutes

15. Resuspend pellet in 15 mL PBS (pH 7.5)

16. Add 15 mL of Vertrel; vortex; centrifuge at 1800*xg* for 30 minutes

17. Save supernatant #1

18. Add 1 volume 0.5 M threonine / 0.14 M NaCl (pH 7.5) to fluorocarbon interface; vortex; centrifuge at 1800*xg* for 30 minutes

19. Combine supernatants #1 and #2

20. Add 8% PEG and 0.3 M NaCl: stir to solution; store overnight 4°C

21. Centrifuge at 14,000*xg* for 15 minutes

22. Resuspend pellets in 2 mL of PBS or freeze pellets at -80°C for future RNA extraction

Results of an initial investigation shows coliphage MS2 was recovered from both tomato and strawberry sauce using an acid-adsorption elution technique as shown in Table 4.4.15. Although the elution only recovered between 10-16% of virus initially seeded in sauce samples, over 100% of the MS2 was recovered following PEG precipitation of the combined eluates. It is not known why recovery of MS2 was low in elution and high after PEG concentration and resuspension. Possibly, the viruses had become aggregated and gave reduced infectivity titers. Nevertheless, these results show that increased purification of tomato and strawberry samples by PEG led to increased detectability of MS2. This early trial with a fecal indicator virus, coliphage MS2, was a precursor to

subsequent work attempting to show that other viruses, like HAV, could be recovered from tomato sauce and strawberries using an acid-adsorption elution method.

Table 4.4.8 Recovery of coliphage MS2 from TS and SS using the acid-adsorption elution method from Figure 4.4.11

Sample	PFUs of MS2 inoculated [a]	PFUs of MS2 Recovered by Step			
		Eluate #1	Eluate #2	Eluates 1+2 (% recovered)	PEG of eluates 1+2 (% recovered)
Tomato sauce	7.1	6.0	5.5	6.1 (10%)	7.5 (100%)
Strawberry sauce	7.1	5.9	6.0	6.3 (16%)	7.4 (100%)

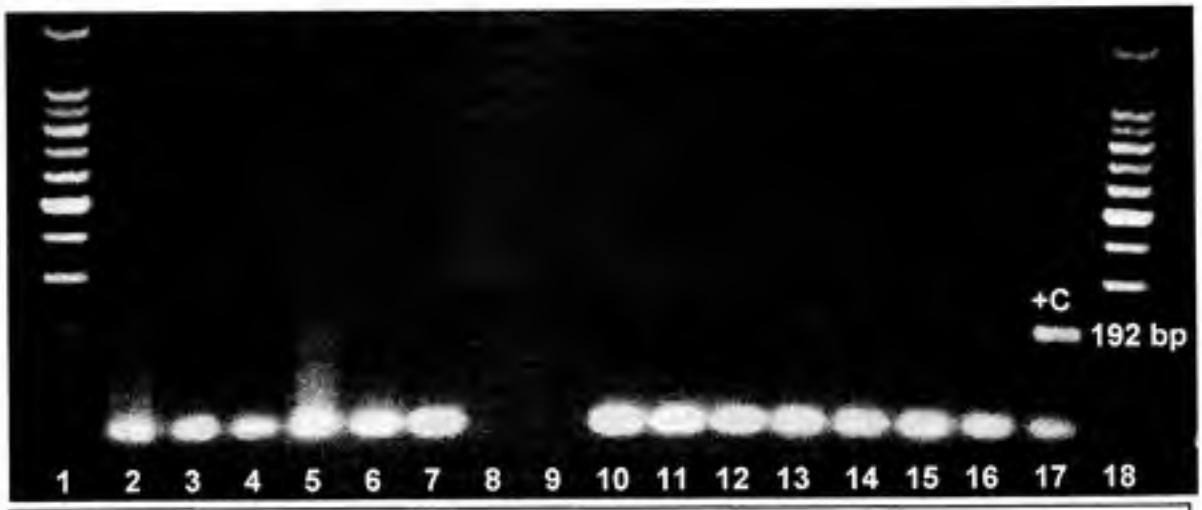
[a] 7.1 log10 PFU coliphage MS2 added to 15 mL (~31 g) of TS or 15 mL SBH (~28 g) for final concentrations of 5.7 log10 PFU/mL (5.4 log10 PFU/g) and 5.7 log10 PFU/mL (5.5 log10 PFU/g), respectively

The acid-adsorption elution technique shown in Figure 4.4.10 was then used to recover HAV from food samples, followed by chemical extraction of RNA and detection by OneStep RT-PCR. Samples were tested with 100 and 1000 PFU added to food samples. As shown in Figure 4.4.12, HAV RNA was not detected in tomato sauce or in oyster samples inoculated initially with 100 PFU, but a faint band of the appropriate size can be seen for the strawberry sample, in lane 13 of Figure 4.4.12. Unfortunately, no independent conformation, like southern blot hybridization, was completed to validate the presumptive 192 nucleic acid band as positive for HAV RNA in the strawberry sample. To benchmark results for the 100 PFU input experiment, assuming 100% recovery, an estimate for the expected recovery in gel electrophoresis bands of undilute samples is 0.35 PFU, 0.035 PFU in 1:10 diluted samples, and 0.0035 PFU in 1:100 diluted samples. It is likely that there were too few viruses in serial diluted RNA extracts to be amplified by OneStep RT-PCR, and visualized by gel electrophoresis. In later experiments, nested RT-PCR was used to increase amplification of nucleic acids and detection of low copy

numbers of viruses. A lack of detection in undiluted RNA extracts may be due to poor efficiency of HAV recovery, which is supported by recoveries of <10% in a similar acid-adsorption elution method in later work to be described below.

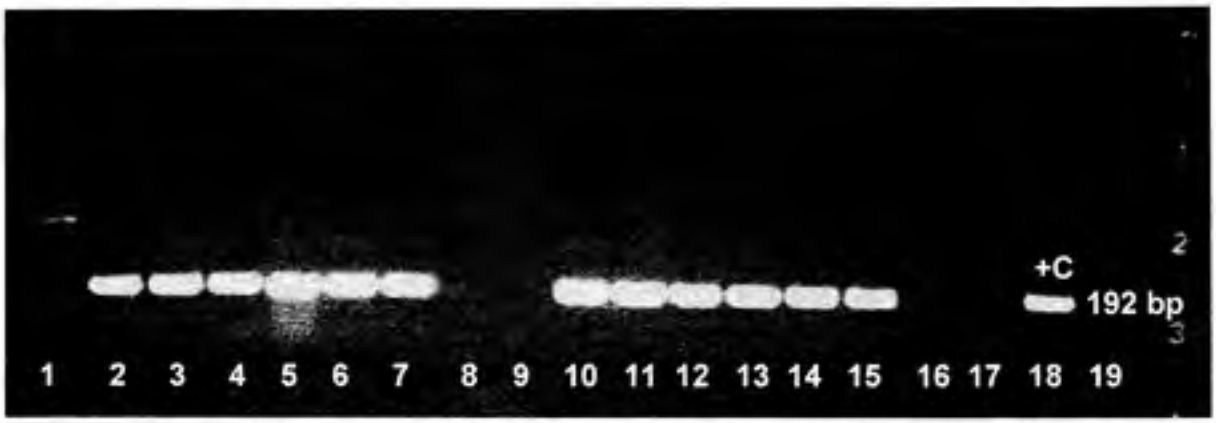
The experiment was repeated using the same method (Figure 4.4.13), but using a higher input (1000 PFU per sample) of HAV. As shown in Figure 4.4.13, HAV RNA was detected in all samples. HAV RNA also was detectable after having been diluted up to 100-fold in deionized water. To benchmark results from the experiment with 1000 PFU inputs, assuming 100% recovery, an estimate for the expected recovery in gel electrophoresis bands from undilute samples is 3.5 PFU, 0.35 PFU in 1:10 diluted samples, and 0.035 PFU in 1:100 diluted samples. With inoculums of 1000 PFU, enough virus was recovered and concentrated as to allow significant dilution of final RNA extracts (up to 100-fold), allowing a reduction of food-based RT-PCR inhibitor through dilution as shown before in Figure 4.4.8. A seeded positive control should have been included to validate the sampling processing method reduces food-based RT-PCR inhibitors, and that basic steps in the method do not yield low recoveries. After learning from this mistake, later experiments did include seeded positive controls.

Figure 4.4.8 OneStep RT-PCR of RNA from HAV recovered from tomato sauce, strawberry sauce, and oyster samples using an acid-adsorption elution method (food samples seeded with 100 PFU or 3900 RTPCRU)



Lane 1: 100 bp marker; **lane 2:** undiluted RNA from HAV recovered from oyster sample #1; **lanes 3 and 4**: 1:10 and 1:100 dilutions of RNA extract from sample shown in lane 2; **lane 5:** undiluted RNA from HAV recovered from oyster sample #2; **lanes 6 and 7:** 1:10 and 1:100 dilutions of RNA extract from sample shown in lane 5; **lanes 8-9:** empty; **lane 10:** undiluted RNA from HAV recovered from tomato sample; **lanes 11 and 12:** 1:10 and 1:100 dilutions of RNA extract from sample shown in lane 10; **lane 13:** undiluted RNA from HAV recovered from strawberry; **lanes 14 and 15:** 1:10 and 1:100 dilutions of RNA extract from sample shown in lane 13; **lane 16:** negative control; **lane 17:** positive control; **lane 18:** 100 bp marker.

Figure 4.4.9 OneStep RT-PCR of RNA from HAV recovered from tomato sauce, strawberry sauce, and oyster samples using an acid-adsorption elution method (food samples seeded with 1000 PFU or 39000 RTPCRU)



Lane 1: 100 bp marker; **lane 2:** undiluted RNA from HAV recovered from oyster sample #1; **lanes 3 and 4**: 1:10 and 1:100 dilutions of RNA extract from sample shown in lane 2; **lane 5:** undiluted RNA from HAV recovered from oyster sample #2; **lanes 6 and 7:** 1:10 and 1:100 dilutions of RNA extract from sample shown in lane 5; **lanes 8-9:** empty; **lane 10:** undiluted RNA from HAV recovered from tomato sample; **lanes 11 and 12:** 1:10 and 1:100 dilutions of RNA extract from sample shown in lane 10; **lane 13:** undiluted RNA from HAV recovered from strawberry; **lanes 14 and 15:** 1:10 and 1:100 dilutions of RNA extract from sample shown in lane 13; **lane 16:** empty; **lane 17:** negative control; **lane 18:** positive control; **lane 19:** 100 bp marker.

Using a shorter acid-adsorption elution method (Figure 4.3.13) modified for recovery of viruses from non-oyster foods, experiments were conducted to study virus recovery, as detected by realtime RT-PCR and nested RT-PCR. Tomato sauce (30 grams) was inoculated with ~1,400 PFU HAV/ gram in the first step (Figure 4.4.14). The final processed sample was split so 1 ml was assayed by cell culture infectivity, and the rest was RNA extracted for use in realtime RT-PCR and nested RT-PCR. Also, two different eluents were used, sample A was eluted with 0.05 M Glycine –0.14 M NaCl (pH 7.5) while sample B was eluted with a more concentrated elution buffer containing 0.75 M Glycine –0.14 M NaCl (pH 7.5).

Figure 4.4.10 Short acid-adsorption elution method to recover HAV from produce

1. Add 7 volumes (vol:vol) water to tomato sauce and strawberry sample (30 grams)
2. Homogenize in Waring Blender on high for 2 min. Pour into 250 mL centrifuge bottle
3. Adjust pH to 4.8-5.0
4. Shake (horizontally) at 100 RPM for 15 min
5. Centrifuge at 2000 *xg* for 20 min; Discard water supernatant
6. Resuspend pellet in 7 vol (vol:vol) 0.05 M glycine / 0.14 M NaCl (pH 7.5) or 0.75 M glycine/0.14 M NaCl (pH 7.5)
7. Add 0.5 vol (vol:vol) Chloroform and shake 2 min
8. Centrifuge at 5000 *xg* for 30 min;
    a. recover supernatant (eluate # 1)
9. Resuspend interface in 50mL of 0.5M threonine / 0.14 M NaCl (pH 7.5)
10. Shake 2 min, centrifuge at 4657*xg* for 20 minutes; recover supernatant (eluate # 2)
11. Combine eluate #1 and eluate #2
12. Add 8% PEG and 0.3 M NaCl: stir to solution; store overnight 4°C
13. Centrifuge 6,000*xg* for 30 minutes
14. Resuspend pellet in3-5 mL PBS (pH 7.5)
15. Add 0.5 vol Chloroform; vortex; centrifuge at 3000 *xg* for 15 min
16. Recover supernatant; off-gas in petri dish
17. Split the sample: 1 ml for infectivity;
    a. the rest for molecular detection
18. RNA extract whole sample (3-5 ml) using Qiagen 4 ml Midi column
19. Isopropanol precipitate RNA extracts with NH_4OAc; Resuspend in 10 ul TE (pH 8.0)
20. Assay 2.5 ul RNA by qRT-PCR and nested RT-PCR

In Tables 4.4.16, 4.4.17, and Figure 4.4.15 are presented the HAV recovery data from one experiment processed by cell culture infectivity, nested RT-PCR and realtime RT-PCR. The % recovery for sample A by molecular detection methods was 2-10%, while sample B had recoveries between 2-3%. Detection of infectious HAV was in the same range as detection of viral genomic RNA by RT-PCR. As previously noted, HAV detection results for infectivity and nested RT-PCR assays are based on endpoint dilutions, while realtime results for HAV RNA detection are based on rates of RNA amplification and assumptions about level of inhibition. Those assumptions are as follows: based on a titration of HAV in experimentally seeded food samples (Figure 4.4.12), realtime RT-PCR could only detect ~1% of the total virus present in undiluted samples (6.25 grams of food per reaction), because of inhibition to realtime RT-PCR. Assuming this is true, virus levels present in undiluted samples A and B are ~100 times more than detected by realtime RT-PCR in Table 4.3.18 (under % recovered column, undiluted samples). Following this assumption, ~900 and ~300 PFU actually could have been present but were undetected in samples 1 and 2, respectively. With an input of 1,400 PFU HAV/ gram, this corresponds to ~10% and ~3% recovery for sample A and B, given in Table 4.3.16.

By cell culture, 4-5% of HAV was recovered in samples after processing (Table 4.3.16). This corresponds to 290 and 354 PFU of HAV recovered in samples A and B, respectively. Also, 4.24×10^4 PFU HAV was inoculated into PBS as positive control at step 12 of the protocol in Figure 4.4.14, as means to track PEG precipitation, PEG resuspension, and chloroform extraction. In the seeded positive control, 642 PFU or 8%

of the HAV inoculum was recovered by cell culture. It is not known why recovery of the seeded positive control was low. This result was from a single trial and may not be representative of true HAV recovery if repeated. It is well proven by many other investigators that high recoveries of HAV are attainably by PEG concentration (further elaborated in the discussion section). A plausible explanation for low HAV recoveries in the PBS positive control could be that virus is lost to an interface during chloroform extraction, or residual chloroform impeded recovery by cell culture, although no cell death was witnessed.

Samples processed by Nested RT-PCR and visualized by gel electrophoresis are shown below (Figure 4.4.15). Tomato sauce samples had no food-based inhibition to nested RT-PCR even in undiluted samples (6.25 grams of tomato sauce per reaction), where 21% of the total 30 g sample was processed. Samples A and B were diluted to the endpoint, giving a final nested band at the 1:1000 dilution. Based on the detection limit of a titration of stock HAV in water (0.02 pfu), and the detection limit of a titration of HAV in tomato sauce (0.2 pfu), the samples each contain ~ 200 PFU HAV. With an initial input of ~1,400 PFU HAV per gram of food, the % recovery for nested RT-PCR samples is slightly more than 2%.

Samples processed by realtime RT-PCR gave different results than that of nested RT-PCR, due to the great susceptibility of realtime detection to food-based inhibitors. Table 4.4.16 shows % recovery, using the standard curve analysis to calculate PFU observed, and dividing PFU observed by PFU expected assuming no inhibition and 100% recovery.

Realtime RT-PCR recovered 0.1% of the initial HAV RNA in sample A and 0.03-0.4% of the initial HAV RNA in sample B. These recoveries may not an accurate representation of the actual amount of HAV present, because food-based inhibitors mask the true level of virus RNA present. The failure to quantify HAV RNA by realtime RT-PCR in Tables 4.4.13, 4.4.16 and Figure 4.4.8 gives 2 important insights; 1) either the method used to recover HAV from tomato sauce do not remove sample inhibitors, which is shown not to be true when amplifying final RNA extracts by nested RT-PCR (Figure 4.4.14), or 2) realtime RT-PCR on a Cepheid Smart Cycler (Sunnyvalle, CA) has significant limitations in its ability to quantify viruses in complex food matrices, such as oysters, tomato sauce, strawberries and possibly other environmental matrices.

Table 4.4.9 Infectivity, nested RT-PCR, and realtime RT-PCR Results for the % recovery of HAV from tomato sauce using a short acid adsorption elution method

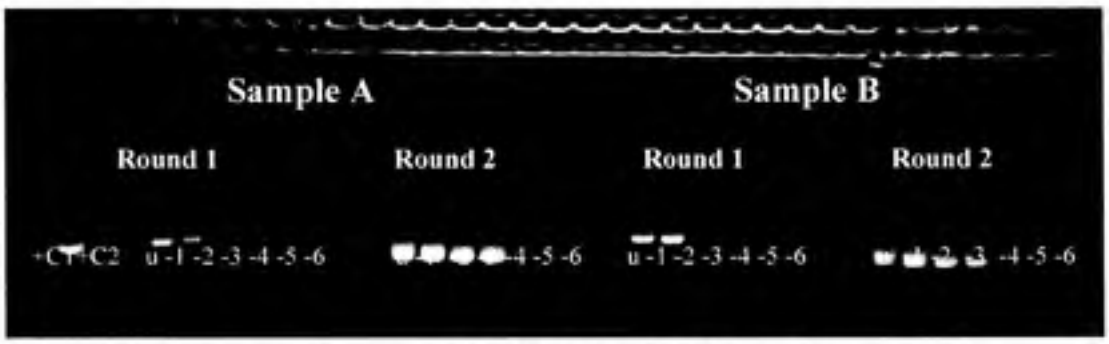
Sample	Food	PFU HAV Input per gram[$]	% Recovered[*]		
			Cell Culture Infectivity	Nested* RT-PCR	Realtime* RT-PCR
A	Tomato sauce	1,400	4%	2%	10%
B	Tomato sauce	1,400	5%	2%	3%
Negative control	Tomato sauce	--	0%	0%	0%

$ Sample size: 30 grams

* % recovered calculated by adjusting for volumes, then dividing the amount of HAV recovered by the amount of HAV inoculated

* based on assumptions made from titration of HAV in food samples

Figure 4.4.11 Nested RT-PCR of HAV in tomato sauce processed by short acid adsorption elution method



Round 1 is synonymous with conventional RT-PCR, Round 2 is the nested assay;+C1,+C2 are Positive controls for Rounds 1& 2; u= undilute; -1 = 1:10 dilution; -2 =1:100 dilution; etc.; Negative controls include a sample negative control and a blank negative control from Round 1, Round 1→ 2, and from Round 2 (on 2nd half of the gel, not shown but were negative).

Table 4.4.10 Realtime RT-PCR to detect hepatitis A virus recovered from tomato sauce by a short acid adsorption elution method

Sample (eluate)	Food (tomato sauce) Total	Per reaction	Realtime RT-PCR Dilution	Ct value	PFU expected	PFU observed	% recovered
A	30 g	6.25 g	Undlilute	35.73	291.7	8.636	0.1%
(0.05 M Glycine-		0.625 g	1:10	38.63	29.17	0.975	0.1%
0.14 M NaCl)		0.0625 g	1;100	42.4	2.917	ND*	0
		0.00625 g	1;1000	ND	0.2917	ND	0
B	30g	6.25 g	Undlilute	37.13	291.7	3.053	0.03%
(0.75 M Glycine-		0.625 g	1:10	37.17	29.17	2.992	0.3%
01.14 M NaCl)		0.0625 g	1;100	39.85	2.917	0.39	0.4%
		0.00625 g	1;1000	ND	0.2917	ND	0
Negative Control	30 g	6.25 g	Undlilute	ND	0	ND	0
Positive Control	In 210 ml PBS		undilute	37.68	16,960	1.99	0.012%
	"		1:10	39.61	1,696	0.468	0.03%
	"		1:100	ND	169.6	ND	0
	"		1:1000	ND	16.96	ND	0

After these somewhat successful applications of a short acid-adsorption elution method (Figure 4.4.14) to the recovery of high titer HAV inoculums in tomato sauce, realtime

RT-PCR was then abandoned for low level quantification of viruses in foods. Further experiments were performed to assess the lower detection limit of the protocol using nested RT-PCR detection. In a series of experiments, serial log ten dilutions of HAV were inoculated into tomato sauce samples, in triplicate. Each low inoculum, 14, 1.4, and 0.14 PFU of HAV per gram of tomato sauce, was processed by the short acid adsorption elution method (Figure 4.4.14). If present, HAV RNA was detected by nested RT-PCR. The detection limit of HAV RNA in tomato sauce is 0.2 PFU per reaction, when amplified by nested RT-PCR in a single trial experiment shown in Figure 4.4.9, although more trials are needed to validate this detection limit. Data in Table 4.4.18 shows 14 PFU per gram of food was the lowest inoculum concentration able to be detected by nested RT-PCR. All three samples spiked at the 14 PFU concentration were positive for HAV RNA at the 1:10 dilution, which corresponds to a 10% recovery. The six samples with ≤ 1.4 PFU of HAV per gram of tomato sauce were not detected as positive by nested RT-PCR. Inoculums of 1.4 PFU were probably at the detection limit of nested RT-PCR.

Another series of experiments, similar to those above, were conducted to evaluate the lower detection limit of HAV detection in strawberries processed by short acid-adsorption elution and analyzed by nested RT-PCR. The detection limit of HAV RNA in strawberries is likely to be 0.02 PFU per reaction, when amplified by nested RT-PCR in a single trial experiment shown in Figure 4.4.10, although more trials are needed to validate this detection limit. As shown in Table 4.4.19, none of the 9 samples inoculated with ≤ 21 PFU of HAV per gram of strawberry sauce (3 samples each with 21, 2.1 and 0.21, respectively) were positive by nested RT-PCR. Previous acid-adsorption work in Figure 4.4.12 and Figure 4.4.13, shows the lowest concentration of HAV initially

inoculated in strawberries and still detectable is ~33 PFU per gram. This value was arrived at from analysis of DNA bands on a gel electrophoresis.

Table 4.4.11 Detection limit of HAV in tomato sauce using a short Acid-adsorption elution method

PFU HAV Input per gram of tomato sauce (30 grams total)	Expected PFU per reaction			% Recovered	Replicates positive by nested RT-PCR (n = 3)
	Undilute	1:10	1:100		
14	106	10.6	1.06	10% *	3/3
1.4	10.6	1.06	0.106	<<0.2 PFU	0/3
0.14	1.06	0.106	0.0106	<<0.2 PFU	0/3

*HAV RNA was detected at 1:10 dilution in three replicates;

Table 4.4.12 Detection limit of HAV in strawberry sauce using a short Acid-adsorption elution method

PFU HAV Input per gram of strawberries (30 grams total)	Expected PFU per reaction			% Recovered	Replicates positive by nested RT-PCR* (n = 3)
	undilute	1:10	1:100		
21	156	15.6	1.56	<<0.02 PFU	0/3
2.1	15.6	1.56	0.156	<<0.02 PFU	0/3
0.21	1.56	0.156	0.0156	<<0.02 PFU	0/3

* The detection limit of HAV RNA in strawberry sauce is likely to be ~ 0.02 PFU per reaction

The work of this section can be summarized in three key points:

1. HAV and MS2 are efficiently acid-adsorbed to produce and the solid particles of sauces during initial steps in acid-adsorption elution. In blended strawberries, 89% (± 5.4%) of infectious HAV and 99% (± 0.3%) of infections MS2 was acid-adsorbed and pelleted with food solids. In tomato sauce, 67 % (± 6.2%) of infectious HAV and 93% (± 1.0%) of infections MS2 were acid-adsorbed and pelleted with food solids. High levels of initial virus adsorption were critical to the success of the acid-adsorption elution method. When key variables in virus adsorption such as sample pH, sample conductivity, and centrifugation force were manipulated, no single modification gave significantly higher recoveries. A more systematic study of virus adsorption may further improve virus adsorption over results from this study.

2. PEG precipitation combined with large volume nucleic acid extraction, and nucleic acid concentration are only moderately efficient at concentrating and recovering HAV from tomato sauce and blended strawberries, with a detection limit of 10^4 PFU of initially inoculated HAV in 30 ml of food eluates. A reason for these low recoveries may be clogging in the silica based RNA extraction filter by residual food particulate. When this method was applied by other investigators from CDC and UNC (Jothikumar et al., 2004) to surface waters, HAV was detected at a sensitivity of 1.6 PFU per reaction in 4.0 ml of RNA extracted virus concentrate from water. It is possible that differences in the sample matrices contributed to differences in the effectiveness of the recovery and extraction methods. However, further studies in which recovery, extraction and detection were compared in the matrices side-by-side would be needed to determine this.

3. Nested RT-PCR is preferable to realtime RT-PCR for analyzing (i) highly concentrated food samples; (ii) samples containing higher levels of enzymatic inhibitors; and (iii) samples with low levels of viral contamination. HAV seeding levels lower than ~33 PFU per gram were not detected by conventional RT-PCR in blended strawberries. Although, using nested RT-PCR, all three samples inoculated with concentrations of 14 PFU HAV per gram of initially seeded tomato sauce were detected as positive, with 10% recoveries.

4.5 Sample Handling Time

Handling time is an important consideration when considering a method for routine use or widespread use among many laboratories. The time needed to perform both the acid-adsorption elution methods and alkaline elution methods were documented in Table 4.5.1. Overall, 1.5 to 3 days were needed to both recover and detect viruses in foods. The protocols processed 4 or 6, 30 gram food samples or about 120-180 total grams. Acid-adsorption methods and alkaline elution methods took similar amounts of time to perform. The greatest time savings was gained when performing realtime RT-PCR (1.5 hours) instead of nested RT-PCR (8 hours) (Table 4.5.1). However, as reported above, realtime RT-PCR was less successful in detecting viruses recovered from food matrices by the method so of this study.

Table 4.5.1 Handling time to recover and detect viruses in foods with acid-adsorption elution and alkaline elution protocols.

Step of Protocol	Time to Complete
Virus elution	Acid-adsorption elution, 3-5 hours Alkaline elution, 2-4 hours
Sample elution concentration	Overnight
RNA extraction and RNA concentration	2-6 hours
RT-PCR amplification and detection	Conventional OneStep RT-PCR, 4 hours Nested RT-PCR, 8 hours Realtime RT-PCR 1.5 hours
Total time	1.5 - 3 days

In this study, the ability of the methods to recover and detect high quantities of virus took precedence over the speed at which the protocol could be performed, although handling time is an important aspect to document. With this information, laboratory analsts can better understand the demands of the method, and similarly, supervisors can better judge time to achieve analytical output, sample throughput and costs.

5 Discussion

5.1 Introduction

Of the illnesses caused by known pathogens each year, the Centers for Disease Control and Prevention (CDC) estimates that around 36 % or 13.8 million of those resulted from foodborne transmission (Mead, Slutsker et al. 1999). Estimates are relied upon because most cases of foodborne illnesses, such as gastroenteritis, go unreported due to the lack of an active government reporting system. With surveillance data from 1983-1992, the CDC lists around 5,000 foodborne outbreaks in the United States infecting around 170,000 patients (Bean, Griffin et al. 1990; Bean, Goulding et al. 1996). From the same 1983-1992 surveillance data, if a known etiological agent was determined, 90-92% of the time it was a bacterial pathogen, and only 5-6% was it a viral pathogen (Bean, Griffin et al. 1990; Bean, Goulding et al. 1996). Why then have foodborne viral pathogens been on the rise in the last decade- and are now the etiological agent in a significant portion of outbreaks? Presumably, the nature of foodborne outbreaks have not have changed significantly from those recorded by CDC in the 1980s and early 1990s. But, one important change is that public health laboratories now have the ability through PCR to detect viral genomic information from previously classified "non-bacteria agents." Formerly unidentified illnesses, nearly a third of which are estimated to be foodborne illnesses, may now be correctly categorized for etiology. Estimates since the availability

of PCR methods now predict around 7 of every 10 foodborne illnesses are caused by viral pathogens (Mead, Slutsker et al. 1999).

The application of molecular biology has revolutionized the identification of pathogens causing viral gastroenteritis, viral hepatitis, and other foodborne viral illnesses. Produce eaten fresh such as blueberries and lettuce or frozen fruits like strawberries and raspberries have been linked to viral hepatitis outbreaks from HAV with contamination often originating at the farm level (Rosenblum, Mirkin et al. 1990; Calder, Simmons et al. 2003). Raspberries, salads, and produce have been linked to norovirus gastroenteritis (Gaulin, Frigon et al. 1999; Lopman, Reacher et al. 2003). Prepared foods, such as restaurant foods, hot-dogs, bread, potato salad, sandwich foods, deli meats, and other mixed foods contaminated by foodhandlers have also been implicated in outbreaks of viral hepatitis from HAV (Osterholm, Kantor et al. 1980; Gustafson, Hutcheson et al. 1983; Hayashi, Yagi et al. 1988; Lowry, Levine et al. 1989; Warburton, Wreghitt et al. 1991) and from noroviruses (Daniels, Bergmire-Sweat et al. 2000; Love, Jiang et al. 2002). Environmental contamination by HAV and norovirus in shellfish, and specifically filter-feeding bivalve mollusks is also well documented and has caused shellfish-borne outbreaks (Dienstag, Gust et al. 1976; Desenclose, Klontz et al. 1991; Xu, Li et al. 1992; Anonymous 1997; Shieh, Monroe et al. 2000; Bosch, Sanchez et al. 2001; Furuta, Akiyama et al. 2003).

To systematize outbreak investigation, the CDC has issued a web-based "Outbreak Investigation Toolkit" providing protocols for sample collection, case identification, a

patient questionnaire, and a form for state or local health departments to report foodborne outbreaks (CDC-Foodborne_outbreak_response). During an outbreak investigation, enteric viruses may be recovered from patients stool and genotyped. A viruses' genetic code serves as a marker for tracking contaminated foodstuffs that may be distributed over a large geographic area. Epidemiological evidence can also predict the source and transmission route of the pathogen. Unfortunately, the CDC model lacks advice on how to collect and process foods implicated in viral outbreaks, and this lack of capacity also exists in many state and local health departments (CDC 2003). Few outbreak investigations are ever able to recover viral pathogens from suspected foods, thus the link from food to patient must be epidemiologically and statistically established. Identifying the microbiological content of suspected foods is an important link in the causative chain of transmission, which can verify that the same strain of virus in consumed food was in patients' stool. Developing methods to recover and detect enteric viruses and fecal indicator viruses (as microbial source tracking tools) in suspected contaminated foods is an important step to curtail outbreaks of viral gastroenteritis and viral hepatitis. For example, Berg and colleagues found virally contaminated oysters transmitted norovirus during 8 separate outbreaks of viral gastroenteritis, all traced to overboard discharge of sewage containing noroviruses into oyster-harvesting sites in Louisiana (Berg, Kohn et al. 2000).

5.2 Recovering viruses from foods

A key objective of the present study was to evaluate and develop methods to recover and detect HAV and a fecal indicator virus, the F+ RNA coliphage called MS2, from a wide

range of foods commonly associated with foodborne viral gastroenteritis and viral hepatitis. HAV was chosen because it is a common agent in foodborne outbreaks, it causes a serious systemtic illness, a strain is available (HM175) that replicates in cell culture, and it is good indicator virus for work relevant to the food industry (Koopmans and Duizer 2004). MS2 is a fecal indicator virus chosen because its physical properties and stablility are similar to many enteric viruses, such as HAV, hepatitis E virus, noroviruses, enteroviruses and astroviruses. Foods selected for testing, oysters, tomato sauce, and strawberries, have all been implicated in foodborne outbreaks associated with HAV and/or noroviruses (Anonymous 1990; Niu, Polish et al. 1992; Anonymous 1997; Hutin, Pool et al. 1999). The economic ramifications of foodborne outbreaks in environmentally contaminated, foodhandler contaminated, and industrial contaminated foods highlights the importance of validating the safety of raw, processed, and vended foods. The foods used in this study also have a representative range of properties including sample consistency, pH, moisture, fat, protein, and carbohydrate content, and therefore, they serve as models for other complex food matrices

For the purposes of virological analysis, foods may be characterized as fluids, permeable solids or semisolids, or surface-contaminated solids (Cliver and Grindrod, 1969). Intact produce and foods such as pasta are likely to fall into the latter category, while sauces and oysters could be classified as permeable solids or semisolids. Two general methods have been used to recover viruses from these kinds of foods (Cliver *et al.*, 1983; Cliver *et al.*, 1983a; Cliver *et al.*, 1990). In the first approach, referred to as surface elution, a food sample is washed or rinsed in a liquid medium (the eluant), which is conducive for the

recovery, growth, and/or detection of the target virus. The second general approach for the recovery of viruses from foods homogenizes or macerates a food sample in devices such as a "stomacher" or blender together with a liquid medium such as an eluant or buffer. Such procedures are usually followed by further processing to concentrate viruses within these liquids, followed by techniques to optimize virus detection.

In bench experiments, 25 to 30 gram food sample were inoculated with virus (HAV and/or MS2) and homogenized to mimic internally contaminated foods. Two popular recovery, concentration and purification methods for intact viruses, elution-concentration and acid-adsorption-elution-concentration, were used to recover viruses from internally contaminated foods. Small-scale experiments were employed as proof of concept, while outbreak investigations may analyze 50 to 100 gram samples in an attempt to increase the likelihood of virus detection.

5.2.1 Efficacy of acid-adsorption step

Acid-adsorption is an effective way to concentrate viruses in shellfish and increase sample quality. In this procedure the sample conditions for pH, conductivity and other parameters are adjusted to those at which viruses will adsorb or remain adsorbed to the food tissue solids for their subsequent elution. In one study, 98% of seeded HAV was recovered from acid-adsorbed oyster tissue (Mullendore, Sobsey et al. 2001). We hypothesized that acidic foods (with ambient food pH levels below pH 7) would adsorb viruses as well or better than shellfish during acid-adsorption steps. Experiments then were designed to determine if acid-adsorption could be applied to concentrate viruses

from tomato sauce and strawberries. Applying typical acid-adsorption steps (Figure 4.3.1) we recovered 89% (± 5.4) of infectious HAV, 87% (± 5.6) of HAV nucleic acids, and 99% (±0.3) of infectious coliphage MS2 from blended strawberries. For tomato sauce samples high virus recoveries during acid-adsorption steps were achieved only for coliphage MS2, with 93% (± 1.0) recovered. When HAV was inoculated into tomato sauce, nearly two-thirds of infectious virus (33% (± 6.2)) and 43% (± 12.8) of viral nucleic acids went unrecovered, apparently due to poor adsorption. From these results it was concluded that acid-adsorption steps recover as much or more HAV in strawberry matrices as previously reported oyster matrices (Mullendore, Sobsey et al. 2001), and have acceptably low levels of virus loss. Because of poor recoveries, acid-adsorption steps needed to be further optimized for HAV recovery in tomato sauce.

Further experiments attempted to optimize acid-adsorption of HAV to tomato sauce by varying centrifugation conditions, food pH and/or food conductivity. Recoveries of HAV nucleic acids in tomato sauce pellets were on average 84% (±12%). No single conductivity level (2,000 uS/cm, 4,000 uS/cm or 6,000 uS/cm) gave significantly better recovery of HAV from tomato sauce, even though relatively small volumes of diluent can drastically change tomato sauce conductivity. The percent of HAV recovered from samples adjusted to pH 4.8-5 was not significantly different from the percent HAV recovered from samples at an ambient tomato sauce pH of ~4.2. Of the pH levels tested (pH 4, 5, and 6) at a conductivity of 2,000 uS/cm, no single pH allowed for significantly better recovery of HAV, and overall recovery ranged from 70% to 82%. In similar work, Sobsey and colleagues found that at the same conductivity, pH levels from 4.5 to 6

performed well (58% to >99 % recovery) for acid-adsorption of poliovirus, reovirus, and adenovirus to oyster homogenate (Sobsey, Carrick et al. 1978). Researchers in France also found pH changes from 4 to7 had no effect on poliovirus acid-adsorption to ground beef or mussels (Finance, Villeval et al. 1981). Possibly, if our pH range were increased, a significant difference in adsorption efficiency would be found, as previously shown for oyster homogenate and fish (Sobsey, Carrick et al. 1978; Finance, Villeval et al. 1981).

A variable thought to have an appreciable effect on virus recovery was centrifugation speed. It was assumed that increasing the centrifugation speeds from 2,000*xg* to 4,000*xg* or 6,000*xg* would increase virus recovery by pelleting more of the solids of the virus-food complex. But, doubling or tripling the centrifugal force gave no statistically significant increase in virus recovery. Tomato sauce samples centrifuged at 6,000*xg* were visibly turbid, unlike oyster or strawberry samples clarified by 2,000*xg* spins. Ingredients in store bought tomato sauce like fats, oils, dyes, spices, and other additives left a thick film on the surface of liquid in the centrifuge tube, but not in strawberry samples. From these observations, we hypothesize that low recoveries of HAV resulted from deficiencies such as inadequate adsorption of viruses to tomato sauce solids, HAV association with non-sedimentable food constituents, and generally poor clarification of virus-food solids under the sample and centrifugations conditions employed. Unfortunately, the non-sedimented food solids also appeared to contain high concentrations of RT-PCR inhibitory and cytotoxic elements to HAV detection, which limited the ability to reliably quantify the viruses.

5.2.2 Concentrating and purifying viruses in food eluates

Polyethylene glycol (PEG) precipitation (8% PEG wt/vol, 8,000 molecular weight (MW) – 0.3M NaCl) was used to concentrate and purify viruses in eluates generated in both acid-adsorption elution and elution methods. PEG has been used to effectively recover enteric viruses from a variety of environmental matrices with high efficiency (Lewis and Metcalf 1988). In tomato sauce and strawberries, there was complete (actually over 100%) recovery of inoculated MS2 using acid-adsorption elution and PEG precipitation. In oyster samples, on average 65% (±17) of infectious MS2 was recovered after PEG precipitation of eluted samples. In parallel experiments, only 3% to 20% of infectious HAV (average of 6.7%, ±5.5) was recovered from oyster eluates. The supernatant following the centrifugation of the PEG precipitate was not assayed, so the levels of infectious HAV remaining in the supernatant liquid of PEG precipitation are unknown. Furthermore, an alternative explanation is that the PEG precipitation procedure was efficient, but that infectious HAV was not effectively assayable from the resulting PEG sediment. The methods reported by other investigators for post-PEG precipitation recoveries of HAV range from 2 to 4% in lettuce samples and 2 to 3% in hamburger samples (Leggitt and Jaykus 2000). PEG recovered 25% of HAV from strawberries using 8% PEG (6,000 MW), 18% using 8% PEG (8,000 MW), and 21% using 13% PEG (8,000 MW) (Cromeans, T.L. Sobsey, M.D. personal communication, unpublished work). In oyster eluates, 8% PEG concentrated 10% to 44% of infectious HAV, and 25% to 51% of infectious poliovirus (Jaykus, De Leon et al. 1996); 72% and 77% of infectious HAV (Mullendore, Sobsey et al. 2001); and 97% of HAV, 40% of human rotavirus Wa, 97% of simian rotavirus SA11, and 104% of infectious poliovirus (Lewis and Metcalf 1988). In

the experiments of this study the PEG precipitation methods employed effectively concentrated MS2 from oysters. However, in parallel experiments no more than one-fifth of the initial HAV was recovered by PEG precipitation, based on quantifying virus infectivity.

Because poor PEG precipitation of HAV was observed, as quantified by infectivity, further efforts were made to concentrate and purify HAV in food samples for detection and quantification of viral nucleic acid, which is the analytical method most likely to be applied to field samples of food. The investigated procedure consisted of the combined steps of PEG precipitation, followed by RNA extraction from a 4 ml volume of resuspended PEG precipitate and then alcohol-mediated nucleic acid precipitation to reduce sample volumes 10,000-fold, from 100 ml of food suspension or homogenate to 0.01 ml. RNA extraction and concentration steps were pioneered in this and other laboratories (Jothikumar et al., 2004) Using an acid-adsorption elution method, a dilution series of HAV was inoculated into phosphate buffered saline (PBS) as a positive control, tomato sauce eluate, strawberry eluate, and oyster eluate, then processed by PEG precipitation, RNA extraction, and detection by realtime RT-PCR (Figure 4.4.5, steps 14-23). In PBS samples, inoculums of ≥1,000 PFU HAV could be successfully detected after PEG precipitation followed by RNA extraction and concentration, with HAV recoveries averaging 84% (± 28) (averaging data in Table 4.4.8). Processed in the same manner as PBS samples (Figure 4.4.5, steps 14-23), 20% and 33% of HAV inoculums were recovered from strawberry samples, while only 3% and 12% were recovered from tomato sauce samples (Tables 4.4.10 and 4.4.11). PBS positive controls, tomato sauce,

and strawberry sauce sample all presented little to no inhibition to realtime RT-PCR, and showed that the 10,000-fold sample concentration would not adversely affect virus detection.

Oyster samples processed and assayed by these methods proved challenging to both recovery and detection of HAV. When oyster samples were inoculated with as much as 1 x 10^5 PFU HAV and processed (Figure 4.4.5, steps 14-23), they failed to give positive signals by realtime RT-PCR. PEG pellets from oyster samples were larger than for other foods, and when resuspended would at times clog the RNA extraction spin columns, which could account for low virus recoveries. To address the poor sample quality in oysters, later experiments used chloroform (1:2 vol:vol) during elution of initial food eluates to purify extracts. The characterization of oyster and other food-inhibitors to RT-PCR will be covered further in section 5.3.1.

5.2.3 Comparing recoveries of HAV to coliphage MS2

HAV and coliphage MS2 are similar in size, shape, and type of genetic material; both are non-enveloped, positive-sense single stranded RNA viruses. The HAV genome, at 7.5 kb in length, is more than twice as long as the ~3.5 kb MS2 genome. HAV is an enteric human virus, infecting human and some primate species, while MS2 is a F^+ RNA coliphage from the Leviviridae family that infects *E. coli*. Both viruses can be quantified by their ability to infect a host and create plaques, or by amplification of genetic sequences within the RNA by RT-PCR In bench experiments, coliphage MS2 is used because it is an easy, quick, and model or surrogate for enteric viruses. MS2 has well documented resistance to chemicals, heat, sunlight, ultra-violet light, chlorination, and water and wastewater treatment processes (Grabow 2001). In the field, MS2 is

enumerated from environmental samples, including foods, as a fecal indicator virus (Sobsey, Battigelli et al. 1995; Hsu, Shieh et al. 2002).

In this study, HAV and MS2 were inoculated together in many experiments. Analysis by infectivity shows that HAV and MS2 were eluted from food samples with similar efficiency (Figure 4.2.6 58% ± 32 for HAV; 76% ±16 for MS2), but when later concentrated by PEG precipitation, MS2 was recovered in significantly higher amounts (Figure 4.2.6 17% ± 5.5 for HAV; 65% ±6.9 for MS2). When MS2 was inoculated into tomato sauce or strawberries processed by acid-adsorption elution and assayed for infectivity (Figure 4.4.11), recoveries were ≥ 100% (Table 4.4.15), while infectious HAV was never recovered from produce in amounts greater than 4-5% (Table 4.4.16).

Some of our data from these experiments with both HAV and MS2 suggest that HAV can be detected at lower seeding levels than MS2. However, these data must be interpreted with caution because HAV was detected by RT-PCR but MS2 was only detected by a plaque assay. In all cases, infectious MS2 was more easily recovered from foods in greater amounts than was infectious HAV, regardless of the method. However, the relative recovery of MS2 and HAV was not quantifiable by the same assay methods because only infectivity and not nucleic acid assay methods were employed for MS2. MS2 recoveries can be compared to HAV recoveries in only some steps of the processing and recovery methods of this study due to the limitations of the assay methods employed. Hence, MS2 was a practical and cost effective alternative virus for which to develop or troubleshoot virus recovery methods. However it can not be used to validate human

enteric viral recovery methods for pathogens such as HAV. This is because the assay methods were different and they were not compared for both viruses.

5.3 Detecting viruses

HAV and coliphage MS2 were detected using infectivity assays and HAV was also detected using three molecular techniques: conventional RT-PCR, nested RT-PCR, and realtime RT-PCR. Detecting infectious viruses is useful in determining the efficacy food disinfection (such as pasteurization), and is one way to apply the current study to the food industry. The practical detection limit of HAV in cell culture, using FRHK cells, was 1 plaque per 0.200 ml inoculum on 60 mm cell culture dishes. Coliphage MS2 was assayed by a double agar layer method with a detection limit of 1 plaque per 0.1 ml inoculum on 100 mm dishes. Molecular detection is another more sensitive method to divulge the pathogenic virological content of foods. Molecular detection is useful in foodborne outbreak investigations involving molecular epidemiology, and virus source tracking. Unlike infectivity assays, RT-PCR based molecular detection methods can only provide information on the amount of viral nucleic acids and not information on virus infectivity. The Conventional RT-PCR with a OneStep procedure used 25 µl reaction volume, 2.5 µl of template (sample) RNA, and primers amplifying a 192 bp length segment of the HAV genome connecting the VP1 and VP3 capsid proteins (Schwab et al., 1995). The endpoint of detection of HAV in a stock virus preparation of partially purified cell culture extract was at the 10^{-7} dilution (Figure 4.2.7), corresponding to a detection limit of 0.5 PFU per reaction and a ratio of 38 RTPCR:PFU. The relationship between infectious particles and RT-PCR amplifiable particles estimated in this study is similar to ratios of 25:1(Casteel 2001) and 23:1 (Schwab, 2000) previously reported.

Nested RT-PCR was 1 log more sensitive than conventional RT-PCR for virus detection. Nested first round amplification used a similar OneStep protocol as for conventional RT-PCR, then 1µl of first round product plus a HotStar Taq mastermix for second round "nested" amplification. Primers spanned the conserved VP1-VP3 capsid region of the HAV genome (Hutin, Pool et al. 1999). A lower detection limit of 0.02 PFU per reaction was observed in nested products (Figure 4.3.1). Based on the infectivity of the stock of HAV amplified in nested RT-PCR, and assuming the final visible band in the nested assay contains 1 RT-PCR unit, a ratio of 50 RTPCR:PFU was estimated.

The detection limit of realtime RT-PCR in a Smart Cycler was 0.28 PFU per reaction, similar to conventional RT-PCR and first round products of nested RT-PCR. But, the efficiency of a reaction and the slope of a dilution series of reactions, not the detection limit are more important for generating standard curves and quantifying unknown samples. Standard curves were generated by RT-PCR amplifying 2.5µl to 5µl of serial tenfold dilutions of stock HAV of known concentration in 20 µl reaction volumes. All standard curves were validated by comparison with a "perfect" standard curve having >5 serial tenfold sample dilutions, a reaction efficiency = 1, and a slope coefficient = 1. Standard curves used in this study most nearly approximated "perfect" standard curves, with >4 serial tenfold dilutions, and reaction efficiencies ranging from 0.85 to 1.03, and slope coefficients ranging from 0.96 to 1.00 (Table 4.3.1).

5.3.1 Sample quality and virus detection methods: a study of inhibitors in RT-PCR and cytotoxicity in cell culture

Experiments were designed to characterize food-based inhibitors to RT-PCR and cytotoxicity to cell culture. For example, samples not initially seeded with viruses were generated using both elution and acid-adsorption elution methods, then inoculated with a known quantity of virus, and then further processed for virus detection in parallel with positive control samples to characterize the performance and validate a detection method.

Cytotoxicity was studied during an elution method by adding 2900 PFU HAV to initially unseeded oyster eluates (steps 1-10 in Figure 4.2.8) or initially unseeded, resuspended PEG precipitates (steps 1-19 in Figure 4.2.8). Then, these samples were either diluted in PBS or were further diluted in food eluate or resuspended PEG precipitate of the food, and portions were analyzed by infectivity assay. Food samples diluted in PBS, had no cytotoxicity and allowed for detection of the calculated theoretical or expected virus seeding level for each tenfold dilution (Table 4.2.4). Oyster samples seeded with HAV and diluted in virus free oyster eluates had significant cytotoxicity, such that either plaques were not visible, or counts were well below expected results. Unseeded oyster samples diluted in PBS and amplified by conventional RT-PCR were detected with 1 log greater sensitivity than other samples, although HAV RNA was not detected in any sample containing < 290 PFU in RNA extracts (Table 4.2.4 and Figure 4.2.9).

From these experiments it was concluded that sample quality from alkaline elution methods is so poor as to underestimate virus titers of undiluted samples in both infectivity and RT-PCR assays. Importantly, sample dilution will mitigate cytotoxicity to cell

culture, but not to RT-PCR, presumably because oysters contain stronger inhibitors to the molecular detection method. The theoretical detection limit of cell culture, being 1 plaque forming unit, was more sensitive than conventional RT-PCR for detecting HAV in oysters after recovery by alkaline elution methods. However, from a practical standpoint, it is the nucleic acid methods that will be employed in field sample analysis and for which sensitive and efficient sample processing and virus and viral nucleic recovery, purification and concentration methods need to be developed.

In agreement with the results of previous investigators, foods processed by acid-adsorption elution methods had less food-based inhibitors to RT-PCR than those processed by elution methods (Shieh, Calci et al. 1999), even though acid-adsorption methods involved dramatic (1,000 to 10,000-fold) sample concentrations (reduction in sample volumes). The successful removal of RT-PCR inhibitors in foods was achieved using methods common in plant virology. In a series of experiments, tomato sauce, strawberry, and oyster samples not seeded with viruses were processed by acid-adsorption elution. These unseeded, processed samples were RNA extracted and then seeded with a series of dilutions of HAV RNA for detection by nested and realtime RT-PCR. Nested RT-PCR was less affected by food-based inhibitors and gave 1 log more sensitive detection than did realtime RT-PCR.

When 7.5 grams of food was processed as 5 ul of template in one 25 ul realtime RT-PCR reaction, 1% and 8% of seeded inoculums were detected in tomato sauce and strawberry samples, while using nested RT-PCR for similar undilute sample volumes, virus RNA

was detected with strong bands of amplified DNA (Figures 4.4.9 and 4.4.10). Realtime RT-PCR of HAV in tomato sauce and strawberries showed minimal inhibition when RNA was assayed as 1:10 dilutions in water, but preferably, samples should be processed as serial dilutions from undiluted to the 1:100 dilution (Figure 4.4.8). Oyster samples gave no inhibition to realtime RT-PCR when sample RNA was diluted ≥ 1:100 in water (Table 4.3.9), but samples were not processed in parallel for analysis with nested RT-PCR. Although nested RT-PCR does not quantify virus levels like realtime RT-PCR, nested assays are more sensitive and the molecular detection method of choice when using samples with known PCR inhibitory material. We conclude that new and important information on food-based inhibitors, sample quality, and advances in virus recovery, concentration and purification methods was developed from these studies. Experiments employing sample processing and subsequent realtime RT-PCR titrations of viruses added at later processing steps to these samples was a successful approach. . Furthermore, nowhere in the published literature could we find realtime (RT)PCR used systematically as a predictive tool to analyze sample quality in relation to RT-PCR detection.

5.3.2 Limits of detection of Alkaline elution and Acid-adsorption elution methods

Seeding of HAV and MS2 into oysters, tomato sauce, and blended strawberries at low input levels was employed to determine the lower limits of virus detection by the developed methods. For the alkaline elution methods when 30 grams of blended strawberries or 30 grams of tomato sauce were seeded with ≥ 8,000 PFU HAV, to give a final concentration of ≥ 533 PFU/gram, the added HAV was eluted and PEG concentrated to 2 ml, with 140 ul RNA extracted and 2.5 ul detectable by RT-PCR and

gel electrophoresis. The lower detection limit of HAV for a multiple elution and PEG method was not determined because seeding levels of 1,000 PFU and 100 PFU in tomato sauce, strawberry, and oyster samples gave no detection in conventional OneStep RT-PCR (Figures 4.2.11 and 4.2.12).

Using an acid-adsorption elution method, HAV is recovered, PEG concentrated to 2 ml, 140 ul is RNA extracted, and then 2.5 ul of template is RT-PCR amplified by conventional OneStep RT-PCR. Amplicons from the above method are detected in gel electrophoresis up to the 1:100 fold dilution (Figure 4.4.13) when 1,000 PFU is initially seeded in 30 grams of food at a concentration of 32 PFU/gram in tomato sauce and 36 PFU/gram in homogenized strawberries (Figure 4.4.12). Tenfold lower seeding at 100 PFU HAV in 30 grams of homogenized strawberries (3.6 PFU/gram) gave a weak positive band by gel electrophoresis (but not confirmed by southern hybridization). Using a revised acid adsorption method (Figure 4.4.14) with similar sample elution and PEG concentration, but a 4 ml RNA extraction plus isopropanol nucleic acid precipitation, and 5 ul of template amplified in nested RT-PCR, HAV initially seeded at 420 PFU per 30 grams of tomato sauce (14 PFU/gram), but not lower initial inoculums of 1.4 PFU/gram or 0.14 PFU/gram, was detected by gel electrophoresis. However, strawberry samples inoculated with ≤630 PFU HAV in 30 grams of blended strawberries or (≤ 21 PFU/gram) and processed with the same revised method (Figure 4.4.14) were not detected by nested RT-PCR. Leggitt and Jaykus (2000) found similar lower detection limits in produce, detecting HAV RNA with initial seeding concentrations as low as 20 PFU/gram in initial food sample sizes of 50 grams. As in the present study, Leggitt and

Jaykus (2000) had difficulty with sample quality, having to dilute RNA extracts as much as 1:100 to achieve detection in nested RT-PCR . Work presented at the International Hepatitis Symposium 2002, Atlanta, by Cromeans and colleagues has the lowest detection limit of HAV in produce, with seeding levels of 10 PFU in 283 grams of frozen strawberries (0.035 PFU/gram) detectable by imunocapture RT-PCR (Cromeans et al., personal communication, unpublished material). The method by Cromeans and colleagues is more sensitive at detecting HAV in frozen strawberries than both this studies method for detecting HAV, and any other method used to recover enteric viruses from contaminated foods in outbreaks (Shieh, Calci et al. 1999; Schwab, Neill et al. 2000). However, the acid adsorption method of this study, although not as sensitive, is capable of recovering HAV from a wide range of complex foods including oysters, produce, fruit, and sauces.

In this HAV was recovered and detectable in 25 grams of oysters seeded with as little as 40 PFU HAV/gram using an acid-adsorption elution method followed by conventional RT-PCR amplification. Other researchers using an acid-adsorption elution method have been able to detect lower inoculums, including <10 PFU of seeded HAV in clams (Sunen and Sobsey 1999), and 1 PFU HAV/ gram (or 333 PCR units/gram) initially seeded in oysters (Mullendore, Sobsey et al. 2001). Acid-adsorption elution methods have also been applied successfully in recovering viruses during two recent environmental sampling studies on mollusks in Switzerland and Spain (Beuret, Baumgartner et al. 2003; Sunen, Casas et al. 2004). Even though Beuret and colleages (2003) used 9% of an oyster sample (comprising 5 homogenized oyster stomachs) as template for RT-PCR, a

significant limitation of the environmental monitoring study is that the lower detection limit of the assay was never determined, therefore a baseline for virus recovery cannot be established. Sunen and colleagues did provide a lower detection limit of 600-1200 PCR units per gram of clam. Using 0.8 to 1 gram of sample as template for nested-RT-PCR, only 15 environmentally contaminated samples were analyzed, thus providing little more than a proof-of-concept for the environmental monitoring method (2004).

5.4 Recommendation on practical aspects of processing suspected virally contaminated food samples

Based on the findings of this study, it is recommended that suspected contaminated foods from outbreaks and those from other studies, such as monitoring and surveillance, be collected according to routine sampling procedures outlined in the FDA Bacteriological Analytical Manual (BAM) (FDA 2003) and the International Commission on Microbiological Specifications for Foods (ICMSF 1986). Sampling varies by food-type, with solid, semisolid, viscous, or liquid foods. Samples should be transported to a laboratory in the original storage conditions (frozen, refrigerated, or at room temperature), and analyzed within 24 or 36 hrs for shellfish and refrigerated foods respectively (FDA 2003).

In the methods development efforts of this present study, great lengths were taken to use simple and reliable materials. All food samples, such as strawberries, cherry tomatoes, head lettuce, pasta salad, tomato with meat sauce, and oysters were processed with one unified method and a few selected reagents that are readily available. The methods also employed readily available kits and reagents for nucleic acid extraction and

amplification. Having one method, a limited number of simple reagents and matreails in the form of kits to analyze all virally contaminated food samples is valuable to laboratories. This is because it is easier to learn and use a limited number of methods, reagents and kits than learning and applying multiple and different methods, it reduces processing time for multiple samples, and it provides a simple, unified basis for method validation and characterization of recovery efficiencies. Steps of the method are adjusted to be compatible with available technology, such that centrifugation speeds are limited to spins within the reach of ordinary lab centrifuges. As well, eluents, buffers, reagents, and kits are easy to make or can be purchased from commercial sources (companies).

5.4.1 Handling Time and time to yield results

Handling time and time to yield results are an important response and cost considerations for laboratories processing virally contaminated food samples. Virus recovery and/or sample quality often increase with more person-hours of sample processing. However, there is a need to produce timely results for remedial or further preventive action. With that in mind, the duration of sequential steps for both methods is listed below.

Separating viruses from food-matrix: Acid-adsorption elution takes 3 to 5 hours to process 4 food samples totaling 100 to 400 grams. Alkaline elution requires 2 to 4 hours to processing a similar batch. Surface contaminated produce would require much less time to process, possibly 1-2 hours.

Concentrating and purifying samples: Concentrating sample volumes by polyethylene glycol (PEG) requires a 4°C incubation for either 2 to 4 hours or overnight (24 hours),

followed by a ½ hour centrifugation. Sample purification by fluorocarbon extraction takes an additional 2 hours, but may be performed in parallel with sample elution as a time saving step.

RNA extraction and molecular detection: RNA extraction and concentration takes from 2 to 6 hours, and RT-PCR in a thermocycler lasts 4 hours plus an hour for nucleic acid visualization by ethidium bromide stained gel electrophoresis. Allow an extra 4 hours of handling time, usually on a second day, for a second round nested or hemi-nested RT-PCR and subsequent visualization by gel electrophoresis. Quantitative RT-PCR lasts 90 minutes and may be substituted for conventional RT-PCR and gel electrophoresis visualization.

Summary: A typical alkaline elution or acid-adsorption elution experiment involving 4 samples would take between 10 to 15 hours of hands-on time spanning 2-3 days, if detected by conventional RT-PCR. The same samples processed for analysis by realtime PCR would take only 1½ to 2½ days. Nested PCR would require a total processing and analysis time of 3-4 days. Detecting HAV by cell culture adds an extra 9 to 10 days before results are available. Furthermore, application to field samples would require an advanced method to detect the viruses in inoculated cell cultures, such as an immunoassay or nucleic acid detection method, due to the absence of microscopically visible CPE for a field strain of HAV. Norwalk Virus and other noroviruses can be detected only by RT-PCR because none have been successfully cultured in the laboratory. Comparing the most sensitive technologies, one research analyst could

process twice the number of samples in a little over a week using realtime PCR instead nested PCR. Subjecting food samples to virus recovery methods combined with realtime PCR would give quantitative results for HAV at least a week before cell culture infectivity assays.

5.4.2 Sample number and sample size

Sample number, sampling size, and sampling schemes are statistically driven parameters that allow analysts to make predictions about a larger population based on the results of a sample population. For example, the population may be all strawberries grown at a farm fertilized with nightsoil or sewage, and the public health risk of interest could be virological content of the strawberries. The equation below determines the quantity (n) of strawberries a researcher should sample, based on p = the probability of having 1 contaminated strawberries, and d = the percent of strawberries contaminated (Reimann).

Equation 5.4.1 Equation to determine sample size of a population

$$P = 1 - (1 - d/100)^n$$

Because low levels of enteric viruses exist in environmental matrices like foodstuffs, large sample numbers and/or large sample sizes are required to detect contaminated products with a high level of confidence. If one assumes 10% of the strawberries grown on a farm are contaminated, and we want to be 95% certain of finding a contaminated strawberry if it exists, we should sample 2,995 individual strawberries. If one assumes a higher proportion of contamination, say 35% of strawberries were contaminated, and one still wanted to be 95% certain of finding a contaminated strawberries, the sample size would be 854 strawberries. From this we can assume spot checking viral contamination in strawberry crops or other foods, except possibly certain high risk foods, is

prohibitively expensive and time consuming. Although, given an outbreak situation where an etiological agent and transmission route is identified by epidemiological investigation, testing representative samples from proposed foods would be feasible and useful for confirming the source of exposure.

When deciding upon a method to recover viruses from foods, it is important to consider the number of samples that can be processed, as well as the sample size, concentration factor, and the amount of sample assayed by the detection method (referred to as a percent, such as "0.1% of the sample volume was assayed in cell culture," or as a weight, such as, "cell culture inoculums represented 0.1 gram of sample"). Intuitively, methods that can process larger (e.g., 50 to 100 gram) food samples seem preferable to those processing smaller (e.g., 10 to 50 gram) food samples, but sample weight alone can be misleading. In one study, poliovirus, norovirus, and HAV were spiked then recovered from as much as 100 grams of fruit or vegetables, but only the equivalent of 0.2 grams of sample was assayed in one RT-PCR tube, which corresponds to 0.2 % of the total sample (Dubois, Agier et al. 2002). Surprisingly, a similar trend exists among many food virology studies for which (RT)PCR is the detection method. Leggitt and Jaykus (2000) recovered poliovirus, norovirus, and HAV from 50 gram food samples, then assayed 0.15 grams of hamburgers or 0.15 to 0.015 grams lettuce in one RT-PCR tube, characterizing the viral content in 0.3% to 0.03% of the total sample (2000). When Schwab and colleagues recovered norovirus and HAV from 30 grams of deli meat, 0.06 grams or 0.2% of the total sample was assayed in one RT-PCR tube.

Large or bulk sample sizes are useful to consider for analysis. However, the current study focused on incorporating relatively large portions of the initial sample mass (as grams or ml of food) in the RT-PCR assay. In our method, an entire 30-gram food sample could be processed for virus recovery, RNA extracted using a 4 ml silica-gel-based spin column and concentrated to 10 ul. As much as 25% of the sample, or the equivalent of 7.5 grams of tomato sauce or blended strawberries, could be assayed for virus RNA free of inhibition in one nested RT-PCR tube. This represents about a 2 $\log_{10}$ or 100-fold increase in the assayable fraction of the initial sample, and about a 1 $\log_{10}$ or 10-fold increase in the initial grams of food assayed per reaction, when compared to similar studies using conventional or nested RT-PCR (Leggitt and Jaykus 2000; Schwab, Neill et al. 2000; Dubois, Agier et al. 2002; Hewitt and Greening 2004).

There have been few studies using realtime RT-PCR to detect enteric viruses in foods. When processed samples of tomato sauce and strawberries were assayed by realtime RT-PCR in this present study, the equivalent of 0.75 grams, or 2.5% of an initial 30-gram food sample was processed inhibition-free in one tube. In previous studies by others, 150 times less sample quantity, or the equivalent of 0.005 grams per tube were assayed for viruses by realtime RT-PCR (Hewitt and Greening 2004). Virological methods giving large assayable sample fractions or portions can reduce costs and labor because they more efficiency examine the sample of interest and may use fewer resources to achieve a more statistically sound representation of the incriminated or suspect food than other methods with smaller assayable sample fractions or portions.

5.5 Finds of this Study and the State of the Science

Bench experiments with experimentally seeded food samples document the ability to recover human enteric viruses from many foods including seafood, meats, vegetables, fruits, mixed foods, sauces, and beverages. However, in most cases, methods cannot recover viruses at actual contamination levels because they cannot separate, recover and detect a high percentage of viruses from foods. Recovering enteric viruses from surface contaminated foods is less complicated than from internally contaminated foods, but in either case most methods cannot efficiently concentrate virus eluates for subsequent efficient virus detection (Jaykus 1997). Advances in sensitive enteric virus detection by molecular method that amplify and detect viral nucleic acids have occurred faster than the development and improvement of methods for virus recovery, concentration and purification in food matrices. PCR technology can detect the genome equivalent of 1 virus particle in a clean sample. However, amplification of viral genomic nucleic acid in a food-matrix is still inefficient due to food-based inhibitors to the enzymatic reactions of (RT)PCR.

Few investigations have been able to link the consumption of contaminated foods to viral gastroenteritis outbreaks or viral hepatitis outbreaks, and fewer still have recovered enteric viruses from suspected foods.(Hutin, Pool et al. 1999; Shieh, Calci et al. 1999; Berg, Kohn et al. 2000; Daniels, Bergmire-Sweat et al. 2000; Schwab, Neill et al. 2000). Methods used to recover enteric viruses from foods have detection limits of 0.4 to 4 PFU HAV and 100 to 1,000 particles of norovirus in foods such as seeded deli meats (Schwab, Neill et al. 2000), and 1 PFU/gram of poliovirus seeded in oysters (Shieh,

Calci et al. 1999). These methods are now approaching detection of the levels of viruses that correspond to the virus doses at which there is a high probability of infection, if ingested. The (RT-)PCR methods now available can be used to detect virus-positive food samples, genotype recovered viruses, and thereby attempt to show a genetic link between viruses recovered from patients and viruses recovered from outbreak foods. The entire endeavor is expensive, time consuming, and is now well documented in the literature. These few published works that are success stories, indicate a promising future for the contribution of food virology to public health protection.

6 Conclusions

- Combining multiple elution steps does not yield better results for HAV recovery, and experiments using samples not initially seeded with test viruses demonstrated that oyster material after multiple elution and RNA extraction still contained significant inhibitors to RT-PCR.

- HAV and coliphage MS2 are eluted from oysters with similar and high recoveries with 58% ± 32% for HAV and 76% ±16% for MS2. However, HAV is not concentrated as well as MS2 by polyethylene glycol (PEG) precipitation, with recoveries of 17% ± 5.5% for HAV and 65% ±6.9% for MS2, respectively.

- For semi-solid, partially liquid foods such as tomato and strawberry sauce or oyster tissue homogenate, an acid-adsorption elution procedure is effective for the recovery of human enteric viruses and coliphage MS2. HAV and coliphage MS2 were efficiently acid-adsorbed to the solids of foods for subsequent virus elution. In tomato sauce, 84% (±12%) of HAV nucleic acids, 67(± 6.2) of infectious HAV, and 93% (± 1.0) of infectious MS2 adsorbs to pelleted solids. In blended strawberries, 89% (± 5.4) of infectious HAV, 87% (± 5.6) of HAV nucleic acids, and 99% (±0.3) of infectious MS2 adsorbs to pelleted solids.

- Dissected oysters may be used in analysis and a protocol for dissection developed in this study may be easily learned by laboratory personnel. However, processed samples from dissected oyster digestive systems are more inhibitory to RT-PCR detection of viruses than are processed extracts from whole oysters. Thersefore, dissection may not greatly increase the ability to analyze oysters for virus contamination.

- Manipulation or adjustment of the pH of food matrices or eluates to a target value or range is often required to successfully recover human enteric viruses and coliphage MS2.

- A concentration step is required to reduce the volume of food eluates or extracts containing viruses. Polyethylene glycol (PEG) precipitation and centrifugal ultrafiltration (CU) may be used for this puropose, but CU as tested in this study is limited by the maximum volume it can accomodate. Hence, PEG is recommended as the primary concentration procedure for human enteric viruses in food eluates. Further studies with larger CU units are recommended, as CU recovery of viruses in food eluates and extracts was efficient.

- Increasing the centrifugation speed and time appears to result in improved recovery of HAV from polyethylene glycol-eluate mixtures.

- Dilution ($\leq$ 1:100) of samples prior to chemical extraction of viral RNA reduces the level of inhibition to RT-PCR but also decreases the level of template and undermines the lower limit of detection of viral RNA.

- Chemical extraction of viral RNA is required for detection using conventional or quantitative RT-PCR.

- A lysis buffer (GuSCN) may be used in place of proprietary lysis reagents for larger-volume extraction of viral RNA from concentrated and purified food eluates, and may also be used to directly extract viral RNA directly from PEG precipitates.

- Fluorocarbon extraction was successfully employed for large food sample volumes during sample homogenization, or after PEG concentration to improve sample quality. Although there is a time penalty in the use of these extractions, they can be optimally employed during multiple virus elution steps.

- With alkaline virus elution methods, cell culture was more sensitive at detecting HAV and could detect theoretically 1 PFU. Experimentally, when samples were diluted serially tenfold as low as 2.9 PFU in a 200 ul cell culture inoculum was detected by infectivity. In comparison, conventional RT-PCR could not detect serial tenfold dilutions of < 290 PFU in 140 ul of RNA extract. This may be because sample dilution was able to mitigate cytotoxicity to cell culture more readily than sample dilution reduces inhibitors of RT-PCR amplification.

- Large volume (4ml) viral RNA extraction combined with alcohol precipitation efficiently recovered viral RNA in virus concentrates from produce and fruit samples. Sample volume reduction was 100-fold and sample inhibition to RT-

PCR was reduced to within acceptable levels, allowing analysis of undiluted or tenfold diluted samples of food extracts. Oysters processed by the methods of this study still contained RT-PCR inhibitors when subjected to large volume (4ml) RNA extraction and concentration and required 1:100 dilution for whole oyster extracts or 1:1000 dilution for oyster stomach extracts to be analyzed free of inhibitors in realtime RT-PCR.

- Nested RT-PCR is a more robust method to detect viral RNA in produce and fruit samples than realtime RT-PCR. Nested RT-PCR has a 100-fold lower detection sensitivity in food-based matrices with ~0.02 PFU/ reaction than realtime RT-PCR with ~2 PFU/reaction in realtime RT-PCR. Undiluted food samples are processed in nested RT-PCR with no apparent inhibition when compared to a 10-fold dilution series of samples, while realtime RT-PCR recoveries from undiluted samples are low (5% ±5%) and must be diluted 1:10 for 82 ± 21% recoveries.

- Realtime RT-PCR has some advantages over conventional and nested RT-PCR. Realtime RT-PCR provides rapid quantification of unknowns, was probe confirmed, had a 1 tube closed reaction and detection in ~90 minutes, while conventional and nested RT-PCR required extensive handling time, lengthy thermocycling steps, used qualitative visualization of DNA products by gel electrophoresis, and needed further confirmation by sequencing or southern blot hybridization.

- Realtime RT-PCR will likely to prove to be especially important for environmental detection, when sample volumes must be rapidly concentrated 100 to 10,000 fold to quickly detect low copy number of viruses.

- Realtime RT-PCR is a useful tool to easily determine sample quality and the extent of sample-related inhibitors in food sample concentrates. In analyzing field samples the use of an internal or external positive controls will be crucial to determine the extent of sample-related inhibition of RT-PCR amplification.

- Using the acid-adsorption elution or alkaline elution methods of this study, processing time to recover viruses in foods and to detect viral RNA are estimated to be: 3-4 days by nested RT-PCR , but only 1 ½-2 ½ days using realtime RT-PCR.

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