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Development of a Novel Method for the Rapid Concentration and Detection of Norovirus and Hepatitis A Virus in Foods

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Development of a novel method for the rapid concentration and detection of norovirus and hepatitis A virus in foods

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Thesis Abstract

Noroviruses (NoV) and Hepatitis A viruses (HAV) are the most commonly implicated viruses in foodborne disease. Their transmission is mainly via the fecal-oral route and distribution of contaminated foods has lead to large outbreaks. Thus, it is crucial that contaminated foods be identified promptly. Detection by cell culture is not possible for these viruses so that researchers rely on the reverse transcription polymerase chain reaction (RT-PCR). Prior to detection, however, viruses must be isolated from foods and, as of yet, no one method has been found applicable to a large variety of food matrices. Problems with existing methods include, but are not limited to, the co-extraction of inhibitory molecules and lack of sensitivity. In addition, they are labour-intensive and time consuming. Therefore, the aims of this research project were to develop a novel methodology for the rapid and sensitive isolation and detection of NoV and HAV from select food matrices. In the first part of these studies, TRIzol® reagent and the RT-PCR were used to isolate and detect feline calicivirus (FCV) (a norovirus surrogate) from select artificially-inoculated foods. However, the co-extraction of PCR inhibitors resulted in false-negative results. RNA purification methods were then compared prior to detection and Oligo d (T) beads provided for the best sensitivity. However, virus capture was not sufficiently sensitive to be applied to outbreak situations. In the second part of this thesis, the Pathatrix™ system was applied to the isolation of HAV and NoV from a variety of foods with the use of positively-charged (cationic) magnetic beads. The use of the Pathatrix™ machinery has led to success in detecting a wide range of bacteria from various food matrices, and we were the first to report on its use for the concentration of viruses. When combined with the RT-PCR for detection, the Pathatrix™ system was able to detect HAV from inoculated foods at levels typically found in outbreak situations. Though

results were not as sensitive with NoV-inoculated foods, the methodology was successful at detecting NoV in outbreak food samples. Furthermore, the cationic beads used in the Pathatrix™ system were able to simultaneously isolate two contaminating viruses from food samples. Therefore, the Pathatrix™ methodology reported herein is a very sensitive and rapid method that holds promise for the isolation and detection of HAV and NoV in outbreak situations.

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This thesis is dedicated to my dear family and husband in thanks for their on-going support and encouragement.

List of Abbreviations

ATCC	American Type Culture Collection
bp	base pair
cat	catalogue
CTAB	cetyltrimethyl ammonium bromide
CrFK	Crandell's feline kidney
DDW	double-distilled water
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
EBSS	Earle's balanced salt solution
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FCV	feline calicivirus
FRhK-4	fetal rhesus monkey kidney
g	grams
G(I-V)	genogroup (I-V)
GI	gastrointestinal tract
h	hour
HAV	hepatitis A virus
HBGA	histo-blood group antigen
HEPES	*N*-2-hydroxyethyl piperazine-*N*-2ethanesulfonic acid
hrs	hours
IgG	immunoglobulin G
IgM	immunoglobulin M
M	molar
min	minute
mJ	mega Joules
ml	millilitre
mM	millimolar
MEM	minimum essential medium
MNV-1	murine norovirus 1
NaCl	sodium chloride
NoV	norovirus
NTPase	nucleotide triphosphatase
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDU	PCR-detectable unit
PEG	polyethylene glycol
PFU	plaque-forming units
pI	isoelectric point
RdRp	RNA-dependent RNA polymerase

RNA	ribonucleic acid
RNase	ribonuclease
RTE	ready-to-eat
RT-PCR	reverse-transcription polymerase chain reaction
RT-PCRu	reverse-transcription polymerase chain reaction unit
RV	rotavirus
s	second
spp	species
SDS	sodium dodecyl sulphate
SSC	sodium chloride sodium citrate
TBE	Tris/Borate/EDTA
TCID	tissue culture infectious dose
µg	microgram
µl	microliter
µm	micrometer
U	units
US	United States
UTR	untranslated region
UV	ultraviolet
VPg	genome-linked protein

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Introduction

Daily consumption and exposure to foods makes anyone at risk of contracting one or more of the 250 recognized foodborne (or waterborne) diseases. The incidence of such illnesses is universal, and occurs when ingested foods are contaminated with microorganisms, toxins or chemical agents. Implicated microbiological pathogens include bacteria, viruses, fungi, protozoa, prions and parasites. Depending on the infecting organism and the dose, symptoms can vary from an asymptomatic or mild infection to more severe illnesses such as hepatitis, meningitis, or even death (1). An example of the severity of foodborne (or waterborne) outbreaks can be shown with the tragic events that occurred in Walkerton, Ontario (May 2000) when municipal water became contaminated with *E. coli* O157:H7 and *Campylobacter* from farm manure that ran off into the water supply. Improper monitoring and lack of adequate chlorination led to a massive outbreak where 2,300 people were infected and 7 died (2). Foods are similarly implicated in the propagation of viruses and bacteria and can contribute to both local and international outbreaks through commercial distribution. In 2002, the distribution of norovirus-contaminated oysters harvested from a lagoon in southern France led to an international outbreak that resulted in 327 cases of acute gastroenteritis (3). These outbreaks highlight the ease with which food and water can serve as highly effective vehicles for disease transmission, sometimes leading to outbreaks reaching epidemic proportions.

The high morbidity associated with foodborne outbreaks has a considerable impact not only on public health, but also on the economy. In the US alone, Mead *et al.* (4) estimate that there are 76 million foodborne illnesses that occur each year with

325,000 hospitalizations, 5,000 deaths, and at a cost of almost $23 billion annually. Huge economical burden can be placed on a community during outbreaks due to medical treatment sought, staff on sick leave, and the cost of tracing and controlling the outbreaks (1, 5). Therefore, it is imperative that the source of foodborne outbreaks be identified at early stages of propagation so that financial costs may be minimized and the outbreaks be controlled effectively.

As such, enhanced awareness and monitoring in recent years has led to an increase in reports of foodborne transmission of pathogens due to factors such as increased trafficking of various types of foods between countries, increased mobility of the population leading to enhanced spread of illness, mass production of foods and improved detection techniques. However, while there is heightened awareness towards commonly reported pathogens, investigators must remain vigilant for the appearance of newly emerging pathogens. Thus, the dynamics in food microbiology are rapidly changing where previously recognized pathogens are being monitored and controlled as effectively as possible, while new pathogens are quickly arising and evolving.

1.1 Foodborne Pathogens

Microorganisms involved in foodborne disease share certain characteristics that allow them to survive the harsh conditions of the gastrointestinal tract such as a high acidity in the stomach, the effects of digestive enzymes, defences of the immune system, competition with resident microflora and, in the case of pathogens replicating at a secondary site in the body, the ability to cross the mucosal barrier and to invade foreign tissues. Foodborne pathogens must first overcome these obstacles in order to reach their

site of replication in a viable state so that they can cause infection of the host. Common pathogens transmitted via foods are outlined in Table 1.

Table 1. Common pathogens transmitted via foods.

Bacteria	Viruses	Parasites
Bacillus cereus	Adenovirus	*Cryptosporidium parvum*
Brucella spp.	Astrovirus	*Cyclospora cayetanensis*
Campylobacter spp.	Coronavirus	*Giardia lamblia*
Clostridium perfringens	Echovirus	*Toxoplasma gondii*
Escherichia coli spp. (i.e. O157:H7)	Hepatitis A virus	*Trichinella spiralis*
Listeria monocytogenes	Hepatitis E virus	
Salmonella spp.	Norovirus	
Shigella spp.	Poliovirus	
Staphylococcus (food poisoning)	Rotavirus	
Streptococcus (foodborne)		
Vibrio cholerae		
Vibrio parahaemolyticus		
Vibrio vulnificus		
Yersinia enterocolitica		

Bacteria have long been recognized as agents in foodborne disease, while techniques to characterize and detect viruses are not always readily available (5). In the past few decades, however, the field of virology has rapidly progressed, leading to enhanced recognition of many of these pathogens through the development and improvement of numerous detection methods.

1.2 Foodborne Viruses

Viruses account for approximately 67% of food-related illnesses in the US, compared to 9.7% and 14.2% for salmonella and campylobacter, respectively (4). They are most likely responsible for more of these infections but, due to limitations in their detection in foods, viruses are underreported. Foodborne viruses listed in Table 1 can be

classified according to the resulting disease: 1) viruses causing gastroenteritis, 2) enterically transmitted hepatitis viruses, and 3) viruses replicating in the human intestine but causing illness only after migrating to other organs (i.e., liver, central nervous system) (6).

Unlike their bacterial counterparts, viruses are obligatory intracellular parasites and each has a tropism for one or more specific human, animal or plant cells. Viruses do not encode for all of the components needed to replicate their genomic material (i.e., ribosomes), so that they depend on constituents of specific host cells to complete their infectious cycles (1, 6-8). Although foods do not serve as amplification vectors for viruses, they are ideal vehicles for the delivery of pathogens to the digestive tract. Once ingested, foodborne viruses must remain intact while being exposed to the harsh conditions of the GI tract until they reach the enterocytes. There, viruses replicate their nucleic acids and assemble new virus particles before either being excreted through stools or crossing the mucosal barrier to reach extra-intestinal sites of replication (1, 6, 9). Following replication in the GI tract, the viral titer is high and infected persons can shed large amounts of viral particles in their stools (up to 10^{11} or 10^{8} particles per gram of faeces for some rotaviruses or noroviruses, respectively) (6, 7). Once excreted in stools, viruses can easily be transmitted to other people when proper hygienic measures are not followed (i.e., hand washing).

1.3 Noroviruses

Noroviruses are important foodborne viruses that account for approximately 95% of non-bacterial gastroenteritis outbreaks worldwide (10). These viruses are found in all regions of the world, and although they are more predominant in the winter, their

occurrence can be seen throughout the year. Formerly known as Norwalk-like viruses, noroviruses were first recognized in 1968 following an outbreak of "winter vomiting disease" at an elementary school in Norwalk, Ohio, US (11). Up until the availability of molecular methods to detect, sequence and express noroviruses in the 1990s, there were serious limitations on NoV strain typing which relied on immunological methods based on the use of antibodies and antigens from human clinical samples (12). Since then, however, numerous labs are continuously increasing their ability to characterize NoV strains, both genetically and antigenitically by using molecular methods and generating recombinant capsids (13, 14). Despite these advances, due to the lack of an animal model or cell culture system for human noroviruses, not much is known about norovirus pathogenesis, replication, and host immune response.

1.3.1 Clinical Disease

Noroviruses have a low infectious dose of approximately 10-100 virions (10), thus very little virus is needed to cause illness. Infections are characterized by a very short incubation period of 12-48 h leading to symptoms of projectile vomiting, explosive diarrhea, nausea, low-grade fever, malaise, and abdominal cramping or pain that typically last for 24-48 h (1, 6). The short duration of illness suggests that the innate immune response plays an important role in rapidly clearing norovirus infections (15). Immune responses are short-lived, however, and a person may be susceptible to re-infection with the same strain within six months (16). The adaptive immune system may also be involved in clearing norovirus infections as suggested by prolonged viral shedding (4 months to >2 years) in patients with compromised adaptive immune responses, i.e., transplant patients (15). In all individuals, viral shedding via stools can last up to 3

weeks (17) and symptoms of diarrhea can persist for up to 4 weeks (18, 19). Death is uncommon amongst otherwise healthy persons but the outcome of infection can be more severe in the elderly, the malnourished and those that are immunocompromised. Supportive treatment such as fluid and electrolyte replacement may be necessary to prevent dehydration.

1.3.2 Genetic Classification

The norovirus genus belongs to the family *Caliciviridae*, which is made up of three other genera: lagoviruses, vesiviruses and sapoviruses. Noroviruses and sapoviruses are human caliciviruses, while members of the other two groups are found in animals. Noroviruses are divided into five distinct genogroups (GI-GV), which are further classified into >25 genetic clusters based on the sequence of their RNA-dependent RNA polymerase gene or capsid gene (3).

Viruses belonging to GI, GII and GIV strains infect humans while those found in GIII have been detected in cattle, and a GV strain has recently been discovered in mice (20). Circulating human strains are always classified within either genogroup I or II and, recently, an analysis of norovirus strains isolated from 184 outbreaks in the US (2000-2004) showed that GII NoV strains were most frequently involved in outbreaks (79%) as compared to 19% for GI strains (21). Data from the same study also indicated that strains belonging to genogroup II/genetic cluster 4 (GII/4) have, in recent years, been most often documented as epidemic strains in the US, Ireland, England, the Netherlands, Germany, Japan, New Zealand and Australia. This could be possibly due to their increased environmental stability and virulence (21, 22). This strain first emerged in 1995-1996

and, by 2002-2003, it caused a marked increase in outbreaks, making this an epidemic year for noroviruses (22).

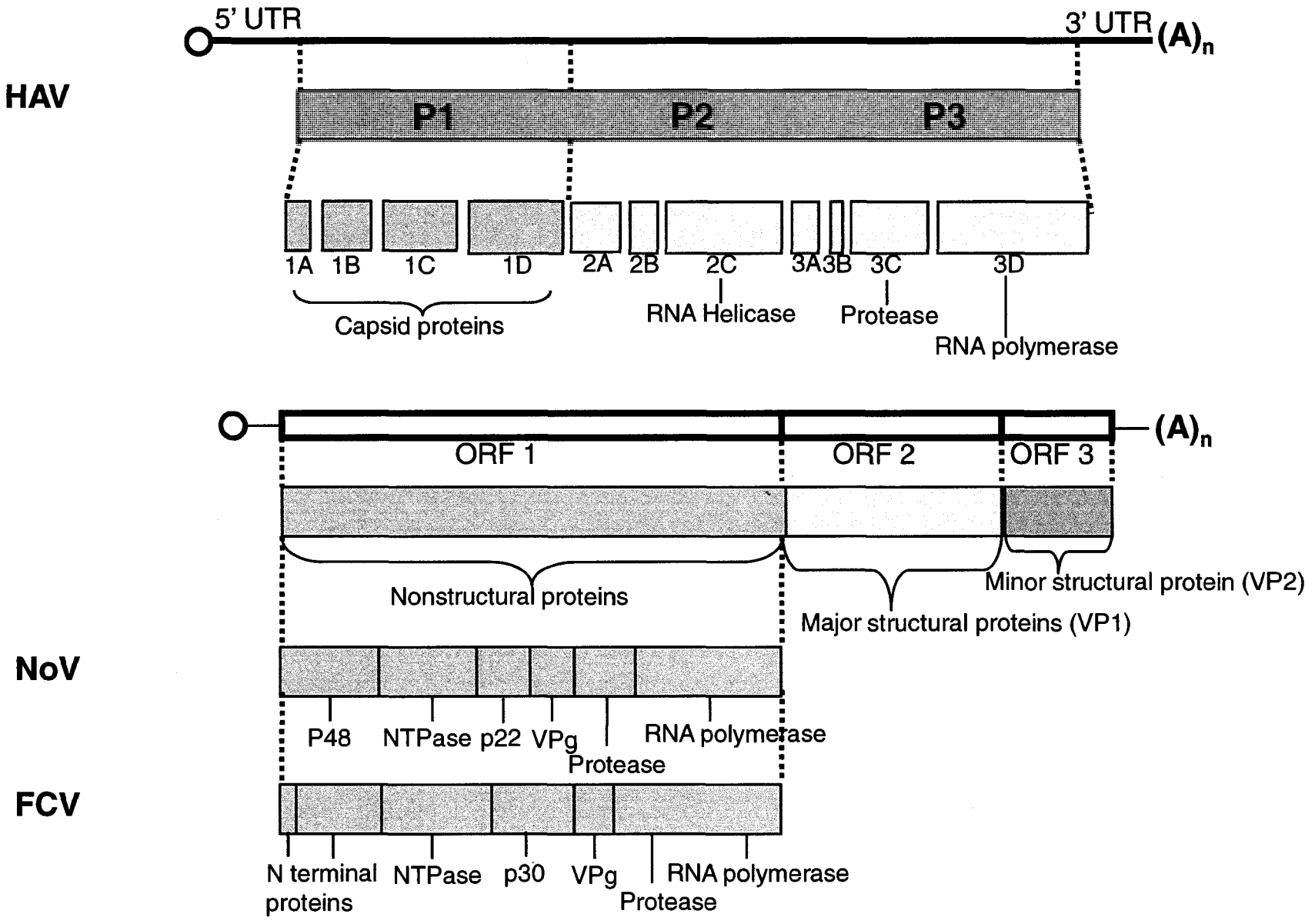
1.3.3 Genome and Proteins

Norovirus particles consist of a single non-enveloped capsid protein of icosahedric symmetry, approximately 27-35 nm in diameter, which contains a single stranded positive-sense RNA genome that is 7.5-7.7 kb in length. The genome is linked to a VPg protein (~15 kDa) at the 5' end and contains a polyadenylated tail at the 3' end. The norovirus genome contains three open reading frames (ORFs) that encode for non-structural proteins (ORF1), a major structural protein (VP1) (ORF2), and a minor structural protein (VP2) (ORF3). (23)

Non-structural proteins are processed co- and post-translationally by the viral 3C-like protease and include (in coding order from the N to C terminus): p48 protein, NTPase, p22 protein, VPg, $3CL^{pro}$, and an RNA-dependent RNA polymerase (Figure 1). Functions for the p22 and p48 proteins have not yet been clearly defined, but there are studies suggesting that the p48 protein may function as a scaffolding protein for the replication complex assembly (24). As in animal caliciviruses, it is probable that VPg proteins are covalently linked to the 5' end of genomic and subgenomic norovirus mRNAs, and are thought to play a role in particle stability (25) or RNA genome packaging (24). The 3C-like protease ($3CL^{pro}$) is similar to the one found in picornaviruses and is named as such for that reason.

The RNA-dependent RNA polymerase (RdRp) encoded by RNA viruses lacks proofreading ability and is more prone to error when replicating its genome, leading to mutations (26). In a study done by Dingle (27), an immunocompromised patient shed

Figure 1. Comparison of the genomic organization and protein products of hepatitis A virus (HAV), norovirus (NoV) and feline calicivirus (FCV). Bolded black lines represent a single genomic molecule with hollow circles depicting the VPg proteins and (A)n representing the poly A tail. For HAV, the genome is translated into a single polyprotein which is posttranslationally cleaved into the capsid and nonstructural proteins. NoV and FCV share a similar genomic organization where the genome is divided into three open reading frames: ORF 1 encodes the nonstructural proteins, ORF 2 encodes the major structural proteins (VP1), and ORF 3 encodes the minor structural protein (VP2).


HAV
5' UTR
3' UTR
(A)n
P1
P2
P3
1A
1B
1C
1D
2A
2B
2C
3A
3B
3C
3D
Capsid proteins
RNA Helicase
Protease
RNA polymerase
NoV
FCV
ORF 1
ORF 2
ORF 3
(A)n
Nonstructural proteins
Major structural proteins (VP1)
Minor structural protein (VP2)
P48
NTPase
p22
VPg
Protease
RNA polymerase
N terminal proteins
NTPase
p30
VPg
Protease
RNA polymerase

norovirus for at least 17 days and the excreted virus mutated at least four times and displayed three microheterogeneities within the 3 kb region sequenced during this time period. Furthermore, amino acid sequence comparisons within the major capsid protein have shown that the similarity between genogroup I and genogroup II noroviruses is less than 50%, while the similarities within a genogroup are ~60% (28). The extreme genetic and antigenic variability seen in the norovirus strains can be due, in part, to inefficiencies in genome replication as well as recombination events (26, 29-31).

Noroviruses are unique amongst animal viruses for assembling their capsids from a single structural protein (32). Capsids are made up of 180 copies of the VP1 protein (90 dimers) that form a T=3 icosahedral virion. Each capsid protein (VP1) folds into two major domains: S for the shell and P for the protruding domain (subdivided into P1 and P2 domains) (33). The P2 domain is the most external part of the capsid and it displays the most variability in sequence, structural size and shape (28, 34). Mutations in this domain are not only thought to be the result of errors in replication, but are also possibly due to immune pressure responses (26). The P2 domain is thought to play an important role in receptor binding and immune reactivity, and is possibly responsible for the susceptibility to infection of persons with ABO histo-blood group antigens (35).

1.3.4 Life cycle

Outcomes from volunteer challenge studies have suggested that a certain subset of individuals is repeatedly susceptible to norovirus infection, while others appear to be resistant (36). Recent studies have recognized that noroviruses use human histo-blood group antigens (HBGAs) as receptors (37-39), so that susceptibility to norovirus infection seems to be suppressed in certain individuals such as nonsecretors who do not have H

antigen receptors (40). Without a tissue culture or animal model, the in vivo site of norovirus replication has not yet been confirmed but, however, based on intestinal biopsies of volunteer challenge subjects, the virus is thought to replicate in cells of the upper gastrointestinal tract (41, 42). Recently, this hypothesis was supported in a study done by Straub *et al.* (43) who showed that GI and GII noroviruses are able to replicate in a physiologically relevant 3-dimensional organoid model of human small intestinal epithelium.

Like other positive-stranded RNA viruses, NoV genomic RNA most likely serves as a template for the synthesis of a minus-strand of RNA which is then replicated to give the genomic plus-strand RNA molecules that are incorporated into progeny virions (44). According to studies with animal caliciviruses, it is thought that noroviruses behave similarly to other positive-stranded RNA viruses by replicating while associated to intracellular membranes (15). At this point, further information on the norovirus life cycle is lacking and will remain unknown until the development of a cell culture system for human noroviruses.

1.4 Hepatitis A Viruses

Hepatitis A virus (HAV) is also an important cause of foodborne disease worldwide (4, 6). In Canada alone, approximately 1000 to 3000 cases of hepatitis A are reported each year, and it is the cause of 25-40% of all acute hepatitis cases (45). The prevalence of HAV infection is highly correlated with socioeconomic status, access to clean water, and sanitation (46, 47). Accordingly, HAV is highly endemic in most Latin American, African, Asian and Middle Eastern nations, while populations in developed countries such as Japan, Australia, New Zealand, Canada, the United States and many

European nations have a low incidence of HAV seropositivity (46). This constitutes a problem for both developing and industrialized nations as HAV infection is common in the former and, for the later, there is a lack of immune tolerance to HAV so that introduction of this virus within the population (i.e., travelers to endemic regions) quickly seeds outbreaks.

1.4.1 Clinical Disease

The HAV incubation period is quite lengthy, ranging from 2-6 weeks (average 28 days), followed by either an asymptomatic or symptomatic (~76-97% of young adults) disease that varies in severity. Children under 3 years of age usually experience an asymptomatic infection while, at the age of 5 years and older, symptomatic infections are common and worsen with age (48). Symptomatic individuals may experience a mild, anicteric illness or fulminant hepatitis and 40-70% of cases are jaundiced (49). Symptoms of a hepatitis A infection typically last for several weeks and include fever, anorexia, nausea, vomiting, diarrhea, myalgia, and malaise concomitant with, or followed by, jaundice, dark-colored urine, or light-colored stools (50). An immune response develops 2-3 weeks after infection and leads to life-long immunity.

Diagnosis of recent infections is based on serological tests that detect IgM antibodies to HAV present 5-10 days before the onset of symptoms (51). IgG antibody detection indicates previous exposure to the virus resulting in a lasting immunity. In healthy adults, the overall case fatality rate is 0.3%, but it is higher among persons aged 50 years of age and older (1.8%) and those with existing chronic liver disease (51). The severity of a HAV infection, particularly for elderly or immunocompromised individuals, renders this virus a serious public health concern. Thus, it is often recommended that

those most at risk to adverse HAV infections (i.e., the elderly) or those that are routinely exposed (i.e., health care workers), be vaccinated against this virus.

1.4.2 Genetic Classification

Hepatitis A virus belongs to the *Picornaviridae* family and is further classified within the *Hepatovirus* genus. HAV differs from other picornaviruses in its nucleotide and amino acid sequences, its difficulty at growing in cell culture systems, and its enhanced resistance to environmental conditions. For these reasons, hepatitis A virus is the sole member of its genus (52) which is further divided into 7 distinct genotypes (I-VII), with types I, II, III and VII found in humans (53). The development of vaccines was facilitated by the fact that HAV only has one serotype (45) and that primates are the only natural host (54). Recently, three human antigenic variants were reported (55, 56).

There are at least 20 different wild-type HAV strains (57) that all adapt poorly to growth in cell culture and their replication is usually very slow and non cytopathic (52). Consequently, researchers commonly use the Australian HM-175 strain in their studies because, following serial blind passages, it has a wide range of cell-culture-adapted mutants. Contrary to noroviruses, the nucleotide sequence of human HAV isolates are highly similar, despite being isolated from different geographical locations (52).

1.4.3 Genome and Proteins

Like other foodborne viruses, hepatitis A particles contain a positive sensed single stranded RNA molecule of approximately 7.5 kb. The non-enveloped capsid is composed of multiple copies of four distinct proteins (VP1-VP4) that assemble into an icosahedron symmetry of 27-32 nm. The uncapped 5' end of the genome contains a noncoding region that is covalently linked to a small VPg protein, while the 3' end

contains a short noncoding region, followed by a polyadenylated tail. The extremities flank a single open reading frame (monocistronic molecule) that encodes for a large polyprotein that is posttranslationally cleaved by a viral protease ($3C^{pro}$) into the various structural and nonstructural proteins (Figure 1).

The open reading frame can be subdivided into three regions: P1 (structural proteins), P2 and P3 (both encoding the nonstructural proteins). The four structural proteins are encoded at the 5' end of the genome, with the VP1 protein being the most variable region of the genome and the 5' UTR being the most conserved region (58). P1 is cleaved into four distinct capsid proteins (VP1-VP4). Sixty copies of VP1-VP3 are needed to assemble each capsid.

The nonstructural proteins include (from the N to C terminals) a viral RNA helicase, protease ($3C^{pro}$), and an RNA-dependent RNA polymerase (RdRp). These proteins are translated immediately after the virus penetrates the cell, as they are needed to synthesize novel RNA molecules and to complete the infectious viral cycle.

1.4.4 Life Cycle

The life cycle of HAV is better characterized than that of the noroviruses due to the existence of cell culture systems for HAV infectivity. Replication studies, vaccine development, and viral propagation have been done in a number of different cell lines (52). The cytolytic variant of HM-175 provides a useful research tool for infectivity and survival studies, because it allows visualization of infection via plaque formation in fetal rhesus monkey kidney cell lines (FRhK-4). However, even when using the lab-adapted HM-175 strain, propagation in cell culture is lengthy and may take several days to weeks to reach maximal titers (52).

The hepatitis A virus life cycle begins when the virus binds (via its VP1 and VP3 capsid proteins) to specific HAV Cr-1 receptors (Ig-like, mucin-like molecules) on the cell surface (52). Conformational changes in the viral anti-receptor (release of VP4) allow the viral genome to be delivered into the cell's cytoplasm with concomitant uncoating of the genomic RNA molecule. Following penetration, the virus uses the cell's translational machinery to translate its genome into a single polyprotein that is posttranslationally cleaved. A viral RNA-dependent RNA polymerase (RdRp) is produced that replicates negative-stranded RNA molecules from the viral genomic RNA (+). In turn, the antisense RNA serves as a template for the generation of novel genomic RNA (+) molecules and/or for the translation of viral structural and/or nonstructural proteins. During replication, nucleic acids are bound to smooth cell membranes. Newly assembled virus particles are released inside membrane-bound packets (1). Novel viruses enter the bloodstream and subsequently migrate to the liver where they replicate in hepatocytes. Damage to infected liver cells is due to the host's immune response rather than pathology caused by the virus (1)

1.5 Transmission Routes

Both NoV and HAV are primarily transmitted by the fecal-oral route, either through person-to-person contact or from ingestion of contaminated food or water. A study done by Blanton *et al.* (21) determined that of the 184 outbreaks caused by noroviruses in the US (2000-2004), 35% were caused by direct contact, 30% were foodborne, 5% were waterborne and no mode of transmission was determined for the remaining 30%. In cases of spread by direct contact, foodborne viruses present on fecally-soiled hands can be transmitted directly between persons or indirectly via

contamination of objects and surfaces (1). It has been shown that NoV and HAV can survive on fomites, i.e., carpets, stainless steel, for prolonged periods of time thereby increasing their chances of transmission (59-63). Moreover, since NoV and HAV display great environmental stability (64-66) and are resistant to widely used disinfectants (67-69), it is not surprising that these viruses cause outbreaks in semi-closed communities where people live in close proximity. Locations most often reported include, but are not limited to, nursing homes, retirement centers, hospitals, schools, restaurants, day-care centers, hotels and cruise ships.

Though direct contact is most often involved in the propagation of enteric viruses, foodborne transmission remains a highly important route. Estimates done in the US by Mead et *al.* (4) indicated that of the 67% of foodborne disease caused by viruses, 40% of NoV infections and 5% of HAV infections originated from foods. Although surveillance data indicate that only a small percentage of HAV cases are transmitted by foods, up to 50% of infections do not have an identified source and many of these may be caused by the ingestion of contaminated foods (50). A large variety of foods have been implicated in the transmission of enteric viruses, with the most commonly reported being ready-to-eat foods (RTE) that do not require any further processing or terminal heating steps prior to consumption (6). Examples of these food items have been well documented and include, but are not limited to, green onions, frozen raspberries, lettuce, deli sandwiches, and bakery goods, with raw molluscan shellfish most often reported (70-76). Foods have the potential of becoming contaminated anywhere throughout the food chain and, unlike their bacterial counterparts, the presence of viruses in foods does not induce spoilage so that there are no visible indications of contamination. Foods become exposed to

pathogens at their site of harvest, with fecally-contaminated growing waters for shellfish and/or with contaminated wastewater used for irrigation or fertilization of produce. Other opportunities for food contamination occur at harvest (infected field harvesters), during processing (production plant workers) or during food preparation (chefs and caterers). At any of the steps mentioned above, foods can come into contact with fecally-soiled surfaces or the hands of infected food handlers or other persons. Since HAV has the ability to survive on experimentally contaminated human hands for several days (unpublished data - (63)), this would allow a food handler sufficient time to unknowingly transfer the virus particles to foods. Consequently, the source of contamination in the majority of documented viral foodborne outbreaks can be traced back to foods handled manually by infected persons rather than by industrial processes (6). Artificially-inoculated fingerpads were shown to transfer >9% of HAV to lettuce during handling (77) and 14-42% of FCV (a norovirus surrogate) to lettuce, ham\turkey, and stainless steel surfaces (78) These data reinforce the need for implementation of proper hygienic practices in the food industry such as continuous washing of hands when handling foods.

Moreover, many studies have reported the persistence of NoV and HAV in select food items thereby increasing the time period in which consumers are at risk of infection. Mattison *et al.* (59) reported that FCV (a norovirus surrogate) could be recovered up until 7 days post-inoculation from lettuce, strawberries, ham and stainless steel when stored at room temperature and 4°C. Transmission from contaminated ice cubes has been well documented for both of these viruses (79-82).

Shellfish (oysters, clams, mussels, cockles) are highly susceptible to viral contamination, as they are filter feeders that concentrate water-contaminating

microorganisms in tissues of their digestive tract. As such, virus levels found in shellfish may be 100-1000X higher than those found in their surrounding water (1). These food commodities are usually consumed raw or slightly cooked and, as opposed to bacterial pathogens, viruses are known to persist in them for prolonged periods of time. NoV in contaminated shellfish has been shown to retain infectivity for more than one month when stored at 4°C or for over 4 months when kept frozen (1). Furthermore, only 7% of norovirus is released from shellfish as compared to a 95% reduction in bacterial levels following depuration practices (83), possibly due to binding on cell-surface carbohydrates in the oyster's digestive tract (84). Most studies have focused on developing detection methods for shellfish, since they have been most often involved in foodborne outbreaks.

Through foodborne transmission, virus-contaminated items have the potential to cause widespread outbreaks when they are consumed by numerous individuals. Table 2 lists outbreaks of norovirus and hepatitis A virus that were attributed to various foods. Regardless of the source of contamination, these outbreaks usually involve a large number of infected persons (70, 71, 73, 85-89). For example, in November 2003, green onions imported from Mexico were consumed at a Chi-chi's restaurant in Pennsylvania, US, and caused a large HAV outbreak which sickened 601 people and resulted in 3 deaths (88).

Table 2. **Foodborne outbreaks associated with hepatitis A virus (HAV) or norovirus (NoV).**

Location	Date	Setting	Implicated foodstuff	Source of contamination[a]	# People infected
HAV					
Pennsylvania, USA (70)	2003	Chi-Chi's restaurant	Green onions	PH or M	601
Massachusetts, USA (71)	2001	Catered event	Sandwiches	P	43
Michigan & Maine, USA (73)	1997	School-lunch programs	Frozen strawberries	PH, DH, or M	213
NoV					
Haute-Loire, France (85)	2005	School cafeteria	Frozen raspberries	PH, DH, M, or P	75
Osaka, Japan (86)	2004	Catering company	Packed lunch boxes	P	91
Massachusetts, USA (87)	2002	Reception Hall	12 wedding cakes	P	332
Netherlands (88)	2001	Restaurant	Dinner rolls	P	231
Helsinki, Finland (89)	1988	Cafeteria	Frozen raspberries	PH or DH	108

[a] Foods were contaminated: Pre-harvest (PH); during harvest (DH); during manufacturing (M); or during preparation (P).

The green onions implicated in the outbreak were thought to have been contaminated prior to arriving at the restaurant. Similarly, oysters contaminated with norovirus during a heavy rainfall were distributed internationally (France and Italy) and caused an outbreak that sickened 327 people (3). Foods contaminated pre-harvest or during manufacturing have an increased chance of being distributed to a larger number of individuals and/or causing outbreaks in several different geographical locations. However, contamination of foods by infected food handlers has also lead to large HAV or NoV outbreaks, though in isolated settings. For example, following a staff buffet lunch, 231 employees in the Netherlands became ill from eating norovirus-contaminated dinner rolls prepared by an infected baker (86). The baker had vomited in the bakery sink on the same day that the rolls were prepared and though he washed the sink and his hands, virus propagation still ensued. Thus, foods have the ability to serve as highly effective vehicles for the rapid and widespread propagation of viruses.

Though respiratory spread is unlikely (90), reports of norovirus transmission via the aerosolization of vomitus particles has been documented (90, 91) and may be important in secondary cases of norovirus infection. It has been estimated that over 30 million virus particles can be liberated during a vomiting episode and that these particles can be inhaled and subsequently ingested, causing spread of the illness (92). Marks *et al.* (90) reported an incident where a restaurant patron vomited onto a polished wooden floor, leading to norovirus infections in several other diners that reported symptoms of gastroenteritis approximately 36 h after the incident. Foods were eliminated as the route of transmission in this outbreak and patrons most susceptible to infection were seated closest to where vomiting had occurred. With one of the main symptoms of a norovirus

infection being the sudden onset of projectile vomiting, the virus ensures that it will release itself in the environment thereby enhancing its chances of infecting more individuals and continuing its life cycle. For hepatitis A virus, no case of aerosol transmission has been documented, however, transmission through contact with blood or blood products can occur (93-95).

Asymptomatic HAV carriers also play an important role in disease transmission (96). With HAV, viral shedding starts 10-14 days prior to the onset of symptoms, which means that infected individuals are unaware of their disease for at least 2 weeks. Moreover, certain individuals with an asymptomatic norovirus infection can shed virus via stools for up to 3 weeks post-infection (6, 97). Thus, those that are unknowingly infected have the potential to propagate the virus to other persons, foods, fomites, or in the environment. Therefore, the best way to reduce virus transmission in the community is by constantly and systematically following good hygienic practices such as the routine and thorough washing of hands following the use of restroom facilities, etc.

1.6 Virus Concentration and Detection Methods in Foods

Since food commodities are implicated as vehicles of transmission in norovirus and hepatitis A virus outbreaks, it is crucial that contaminated foods be identified promptly. Detection of viruses in foods remains a highly challenging task and, as of yet, no methods have been standardized for the application to a large variety of food matrices in outbreak situations. Since viruses cannot replicate outside the infected host cell, the classic food microbiological methods of cultural enrichment and subsequent plating cannot be used with viruses, as is the case for bacterial pathogens. Therefore, novel molecular and/or biochemical techniques need to be developed for their detection.

For many viruses, detection may be based either on electron microscopy visualization or on the observation of cytopathic plaques formed when it is inoculated onto permissive cell lines. However, the most important foodborne viruses do not grow readily (HAV) or at all (NoV) in cell culture, making detection of these viruses by cell culture not possible. As a result, most researchers and diagnosticians rely on molecular methods of detection such as the RT-PCR (reverse transcriptase PCR). Electron microscopy is still used to visualize viral particles, but this method is not sensitive enough as it requires at least 10^{5-6} viral particles per gram of stool and is labor-intensive (6). Routine ELISA assays are available for the detection of some of the noroviruses; however, the detection limits for these tests also require about 10^5 viral particles per gram of stool suspension (6). In addition, many of the norovirus strains are undetectable when using ELISA methodologies due to their vast phylogenetic diversity. Currently, the use of the RT-PCR is favored as the detection method of choice for viruses in clinical and food matrices (1). Furthermore, sequencing of the norovirus genome in the early 1990s (23) has greatly facilitated the detection of noroviruses in clinical stool samples by allowing researchers to design specific norovirus primers for the PCR.

Prior to detection with the RT-PCR, viruses must be isolated from foods and their nucleic acids extracted and concentrated into smaller volumes (μl). Viral concentration from foods offers many challenges such as the complexity and uniqueness of different food matrices, the large food sample volumes to be tested, and the low levels of virus contaminating foods, e.g., ~20-224 viral particles per 100 g shellfish (98). Most existing concentration procedures consist of multiple steps that result in the isolation of either intact virus particles or viral nucleic acids from food samples. Initially, viruses are

usually eluted from food surfaces using alkaline buffers (99-106) or TRIzol® reagent (100, 107, 108). The use of an alkaline buffer promotes viral detachment from food surfaces, resulting in the suspension of virus particles in the buffer (109). Similarly, TRIzol® reagent, a guanidinium-phenol-based reagent, can be used to elute viruses from food surfaces while concomitantly destroying the viral capsid and releasing nucleic acids. Following viral capsid digestion, a series of additional steps must be followed prior to obtaining viral nucleic acid suspensions. These steps are provided in the manufacturer's instructions and include the use of chloroform to separate particles (i.e., proteins, RNA, DNA) present in the resulting wash suspension, isopropanol to precipitate viral nucleic acids, and 70% ethanol to wash the RNA pellet. In their method, Schwab *et al.* (107) solely used TRIzol® reagent to wash and process NoV-inoculated deli meat samples. On the other hand, Kingsley *et al.* (110) used both an initial elution step with an alkaline buffer and TRIzol® reagent to extract and detect NoV and HAV from artificially-contaminated shellfish. In all cases, the elution of virus (or viral nucleic acids with TRIzol® reagent) from food surfaces is a necessary step in providing a virus-containing eluant which can be further concentrated and purified.

Following elution from food surfaces, the buffer contains not only scattered virus particles, but cellular debris from the food matrices. As such, researchers have used several different methods to concentrate and separate virus particles such as poly-ethylene glycol (PEG) precipitation (99, 102, 104, 105, 111, 112), filtration (106, 112, 113), immunomagnetic bead capture (114), ultracentrifugation (115), or a combination of these methods. For example, Dubois *et al.* (105) developed a method to detect NoV and HAV from fresh and frozen produce. In their procedure, an alkaline buffer containing

3% beef extract was used to elute virus from food surfaces prior to a centrifugation step to remove cellular debris. PEG was then added to the virus-containing supernatant to precipitate virus particles overnight, followed by the addition of chloroform/butanol to the reconstituted pellet to separate intact RNA molecules. Though used often to concentrate virus particles, PEG precipitation is a lengthy procedure that increases food processing time.

In another example of virus concentration, Rzezutka *et al.* (103) used ultracentrifugation to sediment virus particles suspended in buffer following an initial elution step. Briefly, virus-inoculated berry samples were eluted with buffer, centrifuged, and then Catfloc and pectinase were added to the supernatant, prior to ultracentrifugation at speeds of 28,000 x g for 30 min, to further reduce the presence of inhibiting substances. The supernatant was removed and submitted to a second ultracentrifugation step at 235,000 x g for 2 h. Though less time-consuming, this method requires that laboratories have the proper centrifugal machines and apparatus, i.e., centrifuge tubes. Regardless of the concentration method used, the above-mentioned processing steps (viral elution and concentration) are necessary in obtaining small (μl) viral nucleic acid suspensions (used for detection in the RT-PCR) from large contaminated food samples (i.e., 25 g).

Following viral isolation, however, detection is often compromised by the co-extraction of inhibitory molecules present in foods. Inhibitory material found in food matrices includes, but is not limited to, proteins, glycogen, salts, phenolic compounds, lipids and pectin. These substances inhibit enzymatic reactions during nucleic acid amplification (the RT-PCR) so that test sensitivity is reduced and/or false-negatives are

obtained. As such, many groups have had to dilute concentrated food extracts (to dilute inhibitors) prior to RT-PCR and/or use nested PCR to allow virus detection from food commodities such as shellfish (100), deli meats (107, 108), raspberries and strawberries (103), produce (105) as well as lettuce and hamburger (99). Schwab *et al.* (107) reported the presence of co-extracted inhibitory material in final RNA suspensions when developing a method to isolate and detect norovirus from artificially-contaminated deli meat samples. In their procedure, TRIzol® reagent was used to elute NoV from 20 g ham samples. Chloroform was then used to separate the wash retentate into 3 phases (RNA, protein, DNA) and, following a 20-min centrifugation step, the viral RNA-containing upper aqueous layer was retained and precipitated with isopropanol. Following a centrifugation step, the resulting pellet was washed with 70% ethanol prior to being resuspended in RNase-free water. False-negative results were obtained when the RNA extracts were not diluted 100-fold prior to the RT-PCR, indicating the presence of PCR-inhibiting residual food particles. Though amplicons (bands) could be detected following the dilution of RNA extracts, this is not an effective way to reduce sample inhibition, as dilution can lead to false-negative results if the starting concentration of nucleic acids is too low.

To circumvent this problem, a number of researchers have incorporated steps in their protocols that purify extracted nucleic acids prior to RT-PCR detection. Separation of nucleic acids from co-extracted PCR inhibitors has been done with a number of commercially-available reagents such as magnetic beads (110, 114, 116), QIAshredder™ homogenizer (111), cetyltrimethyl ammonium bromide (CTAB) (104, 117, 118), pectinase (103, 113) and other products. For example, Guevremont *et al.* (102) were able

to detect as few as 1 RT-PCR unit of norovirus in 25 g of green onions when using poly d(T) magnetic beads to separate viral nucleic acids from non-specific material present in the extracts. Similarly, Sair *et al.* (111) reduced sample viscosity caused by residual food components with a QIAshredder™ homogenizer spin column (which filters out insoluble debris present in RNA suspensions) and reported detection limits as low as 10 and 100 PFU of HAV per 6 g of hamburger and lettuce, respectively. Nucleic acid purification steps such as these have reduced the need for sample dilution, thus increasing the chances of viral detection when food contamination is low.

Viral concentration methods from foods have predominantly focused on the isolation of enteric viruses from shellfish (110, 118-123). When applied to non-shellfish food commodities, these procedures have had limited success in concentrating noroviruses and/or hepatitis A viruses. In recent years, however, more research has been done on developing novel methods applicable to various types of food products. Gouvea *et al.* (117) were the first to develop a method applicable to non-shellfish food commodities such as orange juice, milk, lettuce, and melon. Their protocol, which included guanidium extraction followed by adsorption of RNA to hydroxyapatite and sequential precipitation with CTAB and ethanol, required the use of several reagents, and was labor-intensive, time-consuming and inefficiently removed residual PCR inhibitors. Consequently, a nested PCR was needed for the detection of the virus.

More recently, Butot *et al.* (113) developed a method for the concentration and detection of HAV, NoV, and rotavirus (RV) from a variety of produce such as berries, lettuce and green onions. Initially, virus was eluted from food surfaces by shaking the samples in an alkaline buffer (glycine-Tris with 1% beef extract, pH 9.5) for 15 min.

Virus concentration was then achieved by filtering the virus-containing buffer through a Falcon tube containing a nylon cell strainer of 40-μm pore size. The pH of the filtrate was adjusted to 7.0 before the final debris was separated from the viral suspension by centrifugation. Viral nucleic acids were extracted with the QIAamp™ viral RNA mini kit and amplified with real-time RT-PCR. PCR inhibition sometimes occurred when applying the procedure to berries, resulting in reduced detection sensitivity and/or false-negative results. The addition of pectinase to the protocol greatly improved detection limits by reducing the co-extraction of PCR inhibitors (i.e., pectin) when processing berries.

Though several researchers have taken interest in developing concentration methods for non-shellfish commodities, most have limitations and cannot be applied to all foodborne outbreaks. As such, many of these applications are time-consuming (requiring > 1 day for results) (99), labor-intensive (105), specific to only a few food items (102, 103, 107), or applicable for the isolation of only a limited number of viruses from foods (108, 124). Many methods have also been shown to cause inactivation/destruction of viral particles during the actual process of extraction due to the nature of the chemical agents used. Moreover, developed protocols must be sufficiently effective at recovering and detecting the low levels of virus particles (~10-100 virions) that contaminate foods during outbreak situations. Thus, though results obtained in certain seeding experiments look promising, the procedures may be insensitive when applied to outbreak food samples. For example, the method of Butot *et al.* (113) mentioned previously was highly effective at recovering low levels of virus from virus-inoculated produce but this method was not tested on other food commodities, nor on

outbreak food samples. Although this method holds promise, it does not guarantee success in an outbreak setting.

Rapid and effective control of foodborne viral outbreaks requires that the implicated food commodities be identified and their distribution stopped immediately. As of yet, no one method is available for the detection of enteric viruses from a large variety of food matrices so that "in house" procedures are usually applied during such situations. Moreover, developed methods are still prone to producing false-negative results when applied to outbreak settings. Therefore, it is essential that future research should focus on continued improvement of existing methods and the development of novel techniques with enhanced sensitivity.

1.7 Pathatrix™ System

The Pathatrix™ system (Matrix MicroScience, Newmarket, UK, http://www.matrixmsci.com) is a novel technology that was developed for the concentration, purification and detection of bacteria from various complex food commodities within hours. Matrix Microscience Inc. has standardized protocols for the isolation of *E. coli* O157, *Salmonella* spp., *Listeria* spp., and *Campylobacter* spp. from foods, and these kits are commercially available through this company.

The Pathatrix™ machine comprises five independent units in which the temperature, run time, and speed of circulation are independently set. The use of a higher temperature while processing foods allows for the simultaneous enrichment and capture of various bacteria. Magnetic beads conjugated to pathogen-specific antibodies (immunomagnetic beads) are forcefully recirculated throughout food samples, specifically binding the pathogen of interest which is then immobilized by a magnet located within the

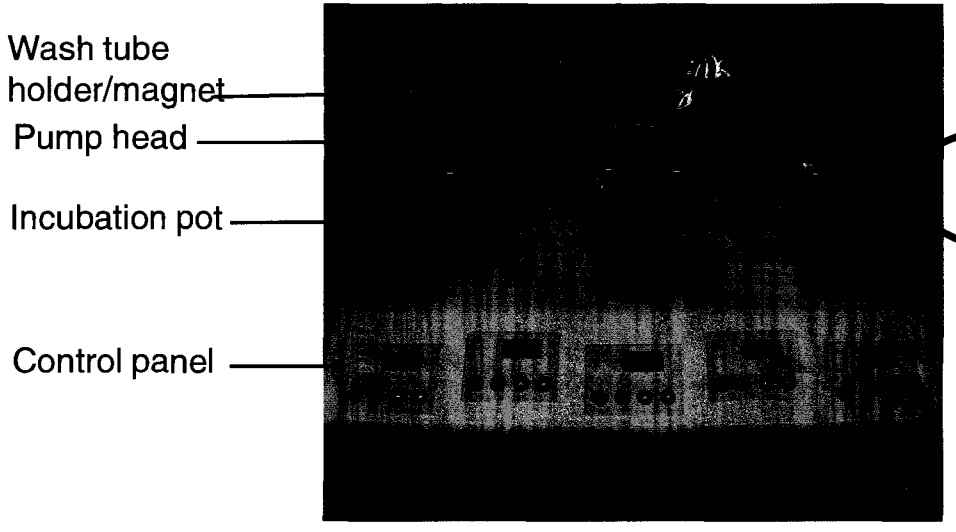
Pathatrix™ equipment (Figure 2). Positively charged magnetic beads (cationic beads) are commercially available (Matrix MicroScience) and can be used in the Pathatrix™ system. Due to their non-specific nature, these beads are particularly useful when antibodies targeting a pathogen of interest are not readily available or when various negatively charged entities are targeted in the same sample (i.e., two viruses). Once isolated, the pathogen(s) can be eluted from the beads, reconstituted in small volumes (50 µl), and identified using a variety of downstream detection applications, including, but not limited to, cultural, direct plating, PCR and ELISA.

Major advantages to the Pathatrix™ system include: large volumes of food samples can be analyzed (up to 25 g of food in 225 ml of buffer) per run, nucleic acids can be directly extracted from virus particles isolated in the resulting sample concentrate, no harsh chemicals are required thereby reducing the chances of viral inactivation, limited reagents are needed so that errors in their preparation are limited, up to 5 samples can be processed simultaneously, it requires minimal handling of samples, and it is an enclosed system not prone to external contamination.

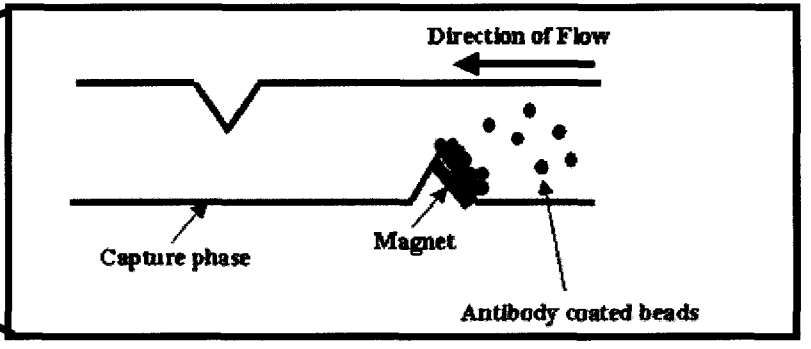
1.8 Norovirus Surrogates

The lack of a cell culture system or animal model for the study of noroviruses has greatly limited research in the characterization of this virus. Hence, most groups rely on the use of a surrogate virus to perform various studies on noroviruses. The most widely used agent is feline calicivirus (FCV), an animal calicivirus belonging to the *Vesivirus* genus. FCV and NoV share numerous biochemical and structural features such as their similar genomic organization (3 open reading frames) (Figure 1) and genomic structure,

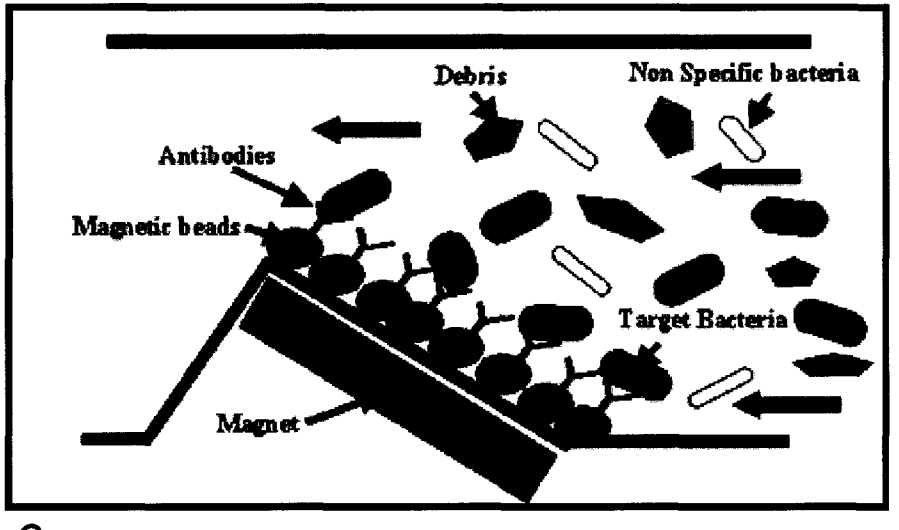
Figure 2. The Pathatrix™ System. The Pathatrix™ machine is comprised of five independently controlled incubation pots (A). Immunomagnetic beads are forcefully recirculated throughout food samples by a pump head (B), and antibody-covered magnetic beads that are bound to the pathogen(s) of interest are captured onto a magnet while non-specific molecules and pathogens are not retained (C). Figures B) and C) were taken from the Matrix MicroScience Inc. website at http://www.matrixmsci.com/.


Wash tube holder/magnet
Pump head
Incubation pot
Control panel

a.


Direction of Flow
Capture phase
Magnet
Antibody coated beads

b.


Debris
Non Specific bacteria
Antibodies
Magnetic beads
Target Bacteria
Magnet

c.

(both have a single-stranded positive sensed RNA genome) as well as their non enveloped icosahedral capsid symmetry.

However, FCV and noroviruses differ in other respects such as their host susceptibility and disease manifestations. Noroviruses induce an enteric disease in humans, whereas FCV causes a respiratory illness in felines. Moreover, unlike NoV, FCV is fragile outside its host and is generally unstable at low pH (125). Cannon et al. (126) showed that FCV was inactivated rapidly at pH extremes of <3 and >9, whereas NoV can survive these harsh conditions (65).

Despite these differences, FCV is a useful surrogate agent of noroviruses in infectivity and survival studies (60, 127-129), because it is easily propagated in cell culture systems and can be rapidly quantified by plaque assay.

An alternative to the use of FCV as a surrogate agent for noroviruses is murine norovirus 1 (MNV-1), which was first described in 2003 (20). It is the only norovirus that replicates in cell culture and in a small animal (20, 130). MNV-1 also has the ability to spread via the fecal-oral route and possibly the respiratory route, which is a characteristic lacking with FCV (15). As such, its use as a surrogate agent of noroviruses may very well surpass that of FCV in the future.

1.9 Thesis Objectives

The aim of this research project was to develop a rapid and universal method for the isolation and detection of NoV and HAV (the most commonly implicated foodborne viruses) from select food matrices of plant and animal origin. The first research objective was multi-faceted and included: optimization of a norovirus concentration method from foods using TRIzol® reagent, the comparison of nucleic acid extraction methods, and the

comparison of several nucleic acid purification methods. Finally, the RT-PCR was used to detect viral RNA using a one-step RT-PCR protocol. Feline calicivirus was used as a surrogate agent for noroviruses throughout these studies.

In the second part of this study, the Pathatrix™ system was used to concentrate norovirus and hepatitis A virus from a variety of select foods with the use of non-specific positively charged magnetic beads (cationic beads). The Pathatrix™ machinery has had great success in detecting a wide range of bacteria from various food matrices (131-136). As of yet, this technology had not been applied to the concentration of viruses from food commodities. This system uses minimal reagents (an elution buffer, a wash buffer, and immunomagnetic beads), accommodates up to five 25 g samples, and requires minimal handling. Consequently, we tested the application of this technology and its ability to simultaneously and rapidly concentrate foodborne viruses from a variety of inoculated foods without the need to modify the methodology for different matrices. The non-specific, positively charged magnetic beads used in conjunction with the Pathatrix™ system could potentially interact with and capture any negatively charged molecules (i.e., the viruses targeted or negatively charged particles found in the food matrix). This is particularly useful when numerous pathogens are contaminating a single sample. Finally, following processing in the Pathatrix™ equipment, the QIAamp™ viral RNA mini-kit was used to extract nucleic acids from captured virus particles. The use of this kit has been widespread in existing viral detection methodologies (103, 113) and the spin columns provided in the kit are designed to isolate extracted nucleic acids from extraneous materials found in the virus suspensions. The virus (es) of interest were

amplified with pathogen-specific primers in the RT-PCR and detected by gel electrophoresis.

Materials and Methods

2.1 Virus Stock and Cell Culture Preparation

Norovirus stocks were prepared from clinical stool samples. Approximately one gram of stool was suspended in 5 ml of 0.9% NaCl, clarified by centrifugation (15 min, 4000 x g, 4°C) (Hettich Rotanta 460R centrifuge), and the supernatant was filtered through a 0.22 μM Steritop filter (Millipore, Ottawa, Canada). Entire stool specimens were processed as such and filtrates belonging to the same stool specimen were pooled. One-ml aliquots of the pooled filtrates were then dispensed into 1.5-ml cryogenic vials (Fisher Scientific, Ottawa, ON) and stored at -84°C until needed.

Seed cultures of Crandell's feline kidney cell line (ATCC #CCL-94) and strain F9 of feline calicivirus (FCV) (ATCC #VR-782) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Methods for the propagation of these cells and the virus have been described previously (137). Briefly, a seed culture of cells was suspended in growth medium containing: Eagle's Minimum Essential Medium (MEM) (Invitrogen Corp., Burlington, ON) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corp.), penicillin/streptomycin (Gibco BRL, Burlington, ON), 7.5% sodium bicarbonate (Sigma-Aldrich Canada Ltd., Oakville, ON) and 100 mM non-essential amino acids (Gibco BRL) (Appendix A) in 75 cm^2 culture flasks (Costar, Canada). Flasks were incubated overnight at 37°C/ 5% CO_2. Subcultures were prepared every 2-3 days by discarding the growth medium from flasks and washing the monolayers with 10 ml of pre-warmed (37°C) Dulbecco's phosphate-buffered saline (PBS; Gibco BRL), pH 7.3. To dislodge cells from the flask walls, 2 ml of 0.05% trypsin-EDTA (Gibco BRL) was added to the monolayers and removed after 20 s prior to

a 10-min incubation at 37°C/5% CO_2. Fresh growth media (10 ml) was then added to cell suspensions and clumps of cells were separated by forceful and repeated pipetting against the interior walls of the flasks. Depending on the split ratio desired (1:3 or 1:2), 3.3 or 5 ml of the cell suspension, respectively, was added to sterile 75 cm^2 culture flasks. Fresh growth media was added to each flask for a total volume of 14 ml. Flasks were incubated at 37°C/5% CO2 until cells formed a monolayer (2-3 days). Cells were maintained and frozen using Eagle's Minimum Essential Medium (MEM) supplemented with 2% fetal bovine serum (FBS) (Appendix A).

The FCV virus pools used were unconcentrated cell harvests that were previously dispensed in 1-ml aliquots and stored at -84°C until used. To prepare a virus pool, confluent monolayers of Crandell's feline kidney (CrFK) cells were grown in 25 cm^2 cell culture flasks, the growth medium was removed by aspiration and 500 µl of the virus inoculum was added to each flask. Flasks were rotated by hand to ensure even dispersal of the inoculated virus onto monolayers of cells prior to, as well as every 15 min during, a 1 h incubation period at 37°C/5% CO_2. Following incubation, 14 ml of maintenance media (MEM + 2% FBS) (Appendix A) was added to flasks and evenly dispersed prior to an overnight incubation period (37°C/5% CO_2). The next day, 80-90% of the cells were destroyed by the virus (as shown by detached, rounded cells) and flasks were frozen and thawed twice at -20°C to release the virus by breaking cell membranes. Flask contents were centrifuged at 4°C, 1,500 x g for 15 min and the virus-containing supernatant was dispensed into 1-ml aliquots and stored at -84°C.

Seed cultures of FRhK-4 (fetal rhesus monkey kidney) cells and hepatitis A virus (HAV) (strain HM-175) were kindly provided by Dr. M.D. Sobsey of the University of

North Carolina (Chapel Hill, NC, USA). Methods for the cultivation and maintenance of the cells and preparation of virus pools have been described previously (63, 138). Briefly, cell cultures were grown in Eagle's Minimum Essential Medium (Invitrogen Corp.) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corp.), 200 mM L-glutamine (Gibco BRL), 100 mM nonessential amino acids (Gibco BRL), penicillin/streptomycin (Gibco BRL), MEM sodium pyruvate solution (Gibco BRL) and 7.5% sodium bicarbonate (Sigma-Aldrich Canada Ltd.) (Appendix A). Protocols for cell propagation were similar to those outlined above for Crandell's feline kidney (CrFK) cells. The media used to maintain FRhK-4 cells was identical to that used for cell growth, but with 2% fetal bovine serum (Appendix A).

The virus pools used were unconcentrated cell harvests prepared by infecting FRhK-4 monolayers at a multiplicity of infection of 0.01. The virus was allowed to adsorb to monolayers for 90 min at 37°C and 5% humidity. Following the adsorption period, maintenance media (MEM + 2% FBS; Appendix A) was added to each flask and infected monolayers were incubated until 70-80% of the monolayers displayed virus cytopathology (4-5 days of incubation). Cultures were then frozen and thawed three times at -20°C. The virus-containing media was removed from the flasks and centrifuged for 10 min at 1,000 x g. The resulting supernatant was dispensed in 1-ml aliquots and stored at -84°C until needed.

2.2 Plaque Assays

Hepatitis A virus and feline calicivirus titers (PFU/ml) were determined by the plaque assay as described by Bidawid *et al.* (114, 137). Briefly, fetal rhesus monkey

kidney (FRhK-4) cells were suspended in growth medium (Appendix A) at a concentration of 5 x 10^5 cells/ml. Portions (1 ml) of this cell suspension were dispensed into each well of a Costar™ 12-well cluster plate (Fisher Scientific), and were allowed to grow into a monolayer by incubating the plates overnight at 37°C/5% CO_2. The next day, the growth medium was discarded and 100 µl of each HAV dilution was inoculated onto a well in triplicate. The inoculated cells were incubated at 37°C for 90 min/5% CO_2, following which 2 ml of an agarose-medium mixture (Appendix B) (63) was added to each well and allowed to solidify. The plates were incubated at 37°C/5% CO_2 for 8 days. The mono-layers were then fixed by the addition of 2 ml of 3.7% formaldehyde to each well, and the plates were left overnight at room temperature. After discarding the formaldehyde, the agarose overlay was removed by gently running warm tap water over the surface of the agarose. The cells were stained by adding 2 ml 0.1% crystal violet (Appendix B) for 30 min, after which the stain was discarded and the plaques were counted visually and recorded as described previously (139). Plaque assays for FCV were done with CrFK cells using the same protocol as with HAV, with the exception that the inoculated plates were incubated for 2 days (137).

2.3 Viral Nucleic Acid Extraction

Three RNA extraction procedures were initially compared to determine the best RNA yield and reverse transcription-polymerase chain reaction (RT-PCR) detection limits. Extraction methods included the QIAamp™ viral RNA extraction kit (Qiagen, Mississauga, ON), TRIzol® reagent (Gibco BRL), or heating for 2 min at 95°C to release nucleic acids from the viral capsid. Either 100 µl (heating process) or 140 µl (QIAamp™ kit and TRIzol®) of virus was initially used for RNA extraction.

The procedure followed for the QIAamp™ kit was according to the manufacturer's instructions. Briefly, 140 µl of virus suspension was lysed under highly denaturing conditions to inactivate RNases with AVL buffer (provided in the kit). Following a 10-min incubation period, ethanol was added to the samples. Solutions were loaded into silica-gel-based QIAamp™ spin columns and centrifuged for 1 min. The RNA bound columns were washed in two steps starting with buffer AW1 and followed by buffer AW2 to get rid of contaminants. Finally, 60 µl of AVE elution buffer was added to each column to elute the RNA from the column.

RNA extraction using TRIzol® reagent was also performed according to the manufacturer's instructions. Briefly, 140 µl of virus was mixed with 800 µl of TRIzol® reagent and incubated for 5 min at room temperature. Following the incubation period, 200 µl of chloroform was added to each of the samples and these were centrifuged at 8,000 x g/15 min. Samples were separated into three phases: an upper RNA-containing aqueous layer, a DNA interface layer, and a protein layer. The upper aqueous layer was retained and the RNA was precipitated with 500 µl of 70% isopropanol (Sigma-Aldrich Canada Ltd.). Following a 15-min centrifugation step, the resulting RNA pellet was washed with 1 ml of 70% ethanol and resuspended in 60 µl of RNase-free Molecular Biology Grade Water (VWR International, Mont-Royal, QC).

The final RNA extraction procedure involved heating 100 µl of the virus suspension at 95°C for 2 min in an Eppendorf thermomixer (VWR International) and immediately placing the sample in a 50% ice bath (50% ice/50% water) for 2 min to avoid nucleic acid degradation caused by heating.

A portion (10 µl) of extracted RNA from each dilution was used in the reverse transcription-PCR (RT-PCR) reaction to determine the positive end-point dilution, which is the highest dilution at which an amplicon of desired size can be detected following RT-PCR amplification and gel electrophoresis. End-point dilutions of the RNA extracted by the various methodologies were compared to determine which of the procedures resulted in the best RNA yield.

2.4 NoV Quantification

To quantify norovirus in stock cultures, 10-fold serial dilutions ranging from 10^0 to 10^{-9} were made in Earle's balanced salt solution (EBSS) (Sigma-Aldrich Canada Ltd.), followed by RNA extraction with the QIAamp™ viral RNA extraction kit (Qiagen) (as described in section 2.3). This kit was selected over other extraction methods because it resulted in a higher RNA yield and the protocol is rapid and user-friendly. Amplicons generated by the RT-PCR were visualized following electrophoresis on a 2% agarose gel in 0.5 X Tris/Borate/EDTA (TBE) buffer and ethidium bromide staining (Appendix C). The virus titer was then determined by end-point dilution where the highest dilution in which a band could be detected represented 1 RT-PCR unit (RT-PCRu) or 1 PCR-detectable unit (PDU). Viral concentration (RT-PCRu per ml) was calculated as the reciprocal of the end-point dilution, taking into consideration the quantity of viral suspension used for the RT-PCR. All amplifications were performed in an Eppendorf Mastercycler gradient (Fisher Scientific) with programs specific to each virus (Table 3).

Table 3. RT-PCR oligonucleotide primer and probe sequences with thermocycling parameters.

Primer and probe sets	Sequence (5'→3')	Location (bp)	Amplicon (bp)	Virus	Reference	Thermocycling conditions
Primers						
CBK-1	GGA GGC GCG ATC TTC AGT AT	102-121	218	FCV	(147)	RT: 42° for 30 min, 95°C for 5 min PCR: 32 cycles each of denaturation at 95°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 1 min and 30 s. Final extension step at 72°C for 5 min.
CBK-2	GCA TAA CTC GTC GGA GGT GT	300-319				
SR33	TGT CAC GAT CTC ATC ATC ACC	4856-4876	123	NV	(148)	
SR46	TGG AAT TCC ATC GCC CAC TGG	4754-4773				
SR48	GTG AAC AGC ATA AAT CAC TGG	4754-4773				
SR50	GTG AAC AGT ATA AAC CAC TGG	4754-4773				
SR52	GTG AAC AGT ATA AAC CAT TGG	4754-4773				
NVp36	ATA AAA GTT GGC ATG AAC A	4487-4505	398	NV	(149)	RT: 42° for 30 min, 95°C for 5 min PCR: 40 cycles each of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. Final extension step at 72°C for 5 min.
NVp110	ACD ATY TCA TCA TCA CCA TA	4865-4884				
HAV1 (upstream)	GTT TTG CTC CTC TTT ACC ATG CTA TG	2167-2192	247	HAV	(150)	RT: 42° for 30 min, 99°C for 5 min, 5°C for 5 min PCR: 40 cycles each of denaturation at 95°C for 1 min, annealing and extension at 60°C for 1 min. Final extension step at 60°C for 7 min
HAV2 (down-stream)	GGA AAT GTC TCA GGT ACT TTC TTT G	2389-2413				
Probes						
SR47d	ATG TCA GGG GAC AGG TTT GT	4804-4823		NV	(148)	
HAV int	TCA ACA ACA GTT TCT ACA GA	2232-2251		HAV	(150)	

2.5 Food Samples

Foods were chosen on the basis of their reported implication in viral outbreaks. Items were purchased from a local supermarket, stored at 4°C and used for testing within two days. Fresh iceberg lettuce, strawberries, and green onions were artificially seeded with HAV, whereas lettuce, strawberries and ham were inoculated with NoV or FCV. Ten-fold serial dilutions of HAV, FCV or NoV stocks were prepared in Earle's balanced salt solution (EBSS) (Sigma-Aldrich Canada Ltd.) and either a 100 µl (100 µl virus in 900 µl EBSS) or a 1-ml (200µl virus in 1.8 ml EBSS) aliquot of each dilution was used to artificially seed each food item. Inoculated foods were left to air-dry in a laminar flow hood for 30 min.

Occasionally, throughout these studies, naturally-contaminated food items were received and processed using the method of concentration being developed at that time. In the spring of 2004, food samples were received from two separate norovirus outbreaks (Ottawa, ON and Guelph, ON) and processed using TRIzol® reagent. The following year, additional food samples implicated in a norovirus outbreak were received from Darnley, Prince Edward Island and were processed using the Pathatrix™ system. Food samples analyzed included shredded cabbage, dry coleslaw, dressed coleslaw, sliced carrot, diced vegetables, dressed potato salad, iceberg lettuce, diced tomato, linguini and mayonnaise.

2.6 Sample Processing with TRIzol® Reagent

In initial studies, the TRIzol® reagent (Gibco BRL) was used to isolate FCV and NoV RNA from select food samples, as per the manufacturer's recommendations (Figure 3). TRIzol® is a mono-phasic solution of phenol and guanidine isothiocyanate, with the

Figure 3. Protocol for the processing of virus-inoculated food samples with TRIzol® reagent. Procedure is derived from the manufacturer's recommendations. Words in bold represent the reagents used and their specified quantities.

1) Inoculate select foods with serial dilutions of virus
2) Incubate for 30 min at room T°
3) Wash with **4ml of TRIzol®**
4) Vortex for 5 min
5) Transfer wash retentates to 50-ml centrifuge tubes
6) Add another **4 ml of TRIzol®** to each sample
7) Vortex for 5 min
8) Pool wash retentates into previous 50-ml centrifuge tubes
9) Centrifuge (8,000 x g, 20 min, 4°)
10) Retain supernatant and discard food pellet
11) Add **1.6 ml of bromochloropropane**
12) Mix for 15 s and incubate at room T° for 3 min
13) Centrifuge (8,000 x g, 20 min, 4°)
14) Retain the upper aqueous phase
15) Add **4 ml of isopropanol** to each sample
16) Mix for 30 s and incubate at room T° for 10 min
17) Centrifuge (8,000 x g, 20 min, 4°)
18) Discard the supernatant
19) Wash the pellet with **8 ml of 70% ethanol**
20) Centrifuge (7,000 x g, 5 min, 4°)
21) Discard the supernatant
22) Air-dry the pellet for 10 min at room T°
23) Resuspend the pellet in **100 -300 µl of RNase-free water**
24) Dissolve pellet by heating for 10 min at 65°C

latter being a strong protein denaturant that allows for release of intact nucleic acids from the viral capsid.

Subsequent to TRIzol® treatment, PCR inhibitors were removed from the nucleic acid extracts by diluting the RNA suspensions 10- and 100-fold or by using commercially-available nucleic acid purification kits such as the QIAshredder™ homogenizer (Qiagen), Dynabeads® Oligo $d(T)_{25}$ (Dynal Biotech Inc., Lake Success, NY), or InstaGene™ Matrix (Biorad Laboratories, Mississauga, ON). Protocols on the use of these products have been outlined in Figure 4.

2.7 Sample Processing with the Pathatrix™

The Pathatrix™ equipment consists of five independent incubation pots that can accommodate up to a 250 ml sample-buffer combination (Figure 5). Twenty-five grams of each food sample is added to a stomacher bag with 225 ml of elution buffer and a standardized amount of magnetic cationic beads (Matrix MicroScience, Inc., Golden, CO, USA). Beads are forcefully circulated throughout the seeded food samples and captured onto a magnet. Isolated bead-bound virus particles can be resuspended and used for downstream applications such as the RT-PCR or the plaque assay.

Each seeded food sample was placed in a stomacher bag (VWR International) containing 225 ml of a pre-warmed (42°C) elution buffer. Initially EBSS (pH 7.2) was used as an elution buffer, whereas glycine buffer (0.5 M glycine, 0.14 M NaCl, pH 8.5, 0.2% Tween 20) (101) was used in later studies. Samples were mixed vigorously by hand for approx. 20 s and each of the samples was either placed directly in one of the five incubation pots of the Pathatrix™ equipment or homogenized for an additional 30 s in a stomacher apparatus prior to being processed. Volumes of 50, 100, 200 or 300 µl of

Figure 4. Protocols for the purification of RNA isolated from foods with TRIzol® reagent. Most procedures have been established according to the manufacturer's recommendations, with only slight modifications.

InstaGene™ Matrix

1) Transfer 30 µl of RNA suspension and 100 µl of InstaGene™ Matrix to a 1.5 ml centrifuge tube;

** Keep InstaGene™ Matrix on magnetic stirrer while taking 100 µl

2) Incubate the tubes in a thermomixer at 56°C for 30 min;

3) Vortex the tubes for 10 s;

4) Incubate the tubes in a thermomixer at 99°C for 10 min;

5) Vortex for 10 s;

6) Centrifuge (12,000 rpm, 4 min, 4°C);

7) Retain the supernatant (~30 µl);

8) Use 10 µl in RT-PCR.

QIAshredder™ Homogenizer

1) Transfer entire RNA suspension to a QIAshredder™ Homogenizer column;

2) Centrifuge (16,000 x g, 2 min, room T°;

3) Retain flow-through and use 10 µl for RT-PCR.

Dynabeads® Oligo $(dT)_{25}$

1) Resuspend RNA pellet (end of TRIzol methodology) in 300 µl of RNase-free water;

2) Add 400 µl of **1X RNA binding buffer** (20 mM Tris-HCl pH 7.5, 1 M NaCl, 2 mM EDTA) to RNA samples;

3) Vortex samples for 30 s;

4) Heat samples in thermomixer at 65°C for 3 min;

5) Add 100 µl of Dynabeads® to each tube;

6) Rock samples gently for 30 s and place in a magnetic tube holder (Dynal Biotech) for 1 min;

7) Gently discard the supernatant;

8) Wash the beads with 500 µl of **2X RNA binding buffer** (40 mM Tris-HCl pH 7.5, 2 M LiCl, 4 mM EDTA);

9) Rotate by hand for 5 min at room T°;

10) Places tubes in the magnetic tube holder for 1 min;

11) Gently discard supernatant;

12) Resuspend beads in 500 µl of **washing buffer** (10 mM Tris-HCl pH 7.5, 0.15 M LiCl, 1 mM EDTA);

13) Place on magnetic tube holder for 1 min;

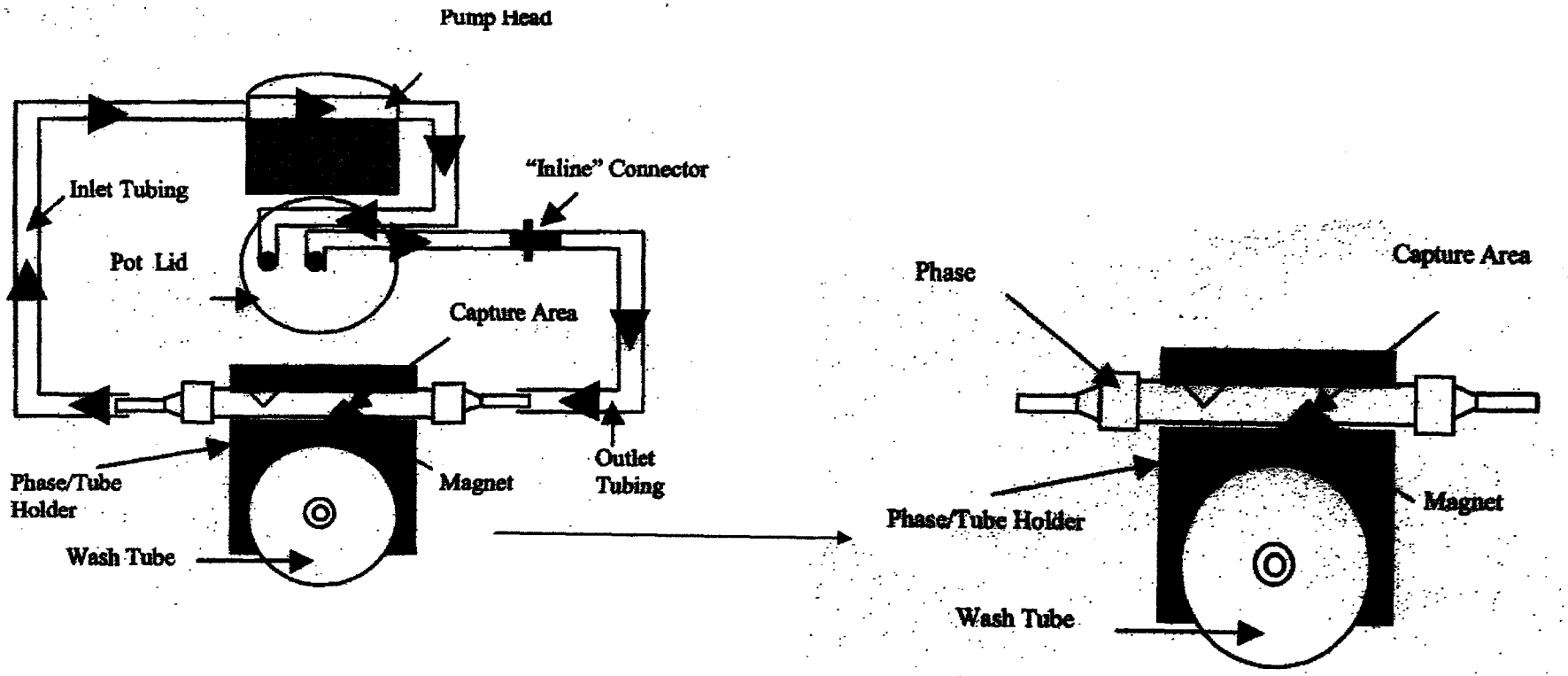
14) Gently discard the supernatant;

15) Repeat steps 12 – 14;

16) Resuspend RNA-bound beads in 100 µl of RNase-free water;

17) Use 10 µl in RT-PCR.

Figure 5. The Pathatrix™ setup. The diagram on the left is an overview of the tubing apparatus setup for each incubation pot on the Pathatrix™ workstation. The diagram on the right is a close-up of where the beads are collected on each incubation pot. Figures were taken from inserts provided with the Pathatrix™ workstation kit.


Pump Head
Inlet Tubing
"Inline" Connector
Pot Lid
Capture Area
Phase/Tube Holder
Magnet
Outlet Tubing
Wash Tube
Phase
Capture Area
Phase/Tube Holder
Magnet
Wash Tube

positively-charged cationic beads (Matrix MicroScience, Inc.) were resuspended by brief vortexing and were added to the stomacher bags prior to attaching the connector tubing of the Pathatrix™ equipment to each incubation pot (Figure 5). The samples were circulated for 30-60 min at 25-30°C in the Pathatrix™ system, and the magnetically-captured beads were washed with 100 ml of pre-warmed phosphate buffered saline (PBS) (20 mM sodium phosphate, 150 mM NaCl, pH 7.2), collected, and re-suspended in 100-140 µl PBS (pH 7.2). Volumes of 100 µl or 140 µl of the bead suspensions were retained for RNA extraction using a heat release process (95°C, 2 min) or the QIAamp™ viral RNA extraction kit (Qiagen), respectively.

Norovirus outbreak food samples received in March 2004 were processed in the Pathatrix™ system using 225 ml of glycine buffer (0.5 M glycine, 0.14 M NaCl, pH 8.5, 0.2% Tween) and 300 µl of cationic beads. After being processed in the equipment for 30 min at room temperature, magnetically-captured beads were washed with 100 ml of PBS buffer (pH 7.2) and resuspended in 140 µl of PBS (pH 7.2) for RNA extraction with the QIAamp™ viral RNA extraction kit.

Studies were performed to assess the efficiency of the cationic beads at binding a target virus while in the presence of other pathogens in foods. Each inoculated lettuce sample was processed in the Pathatrix™ system with 225 ml of glycine buffer (0.5 M glycine, 0.14 M NaCl, pH 8.5, 0.2% Tween 20 buffer) and 200 µl of cationic beads. Following processing, the isolated viruses were subjected to RT-PCR using HAV- and FCV-specific primers.

2.8 Reverse Transcription-PCR (RT-PCR)

The QIAGEN OneStep RT-PCR kit (Qiagen) was used for all RT-PCR reactions. Each reaction contained 10 µl of extracted viral RNA in a total volume of 50 µl reaction mixture, according to the manufacturer's recommendations. Briefly, reaction mixtures contained a proprietary buffer, Omniscript™ Reverse Transcriptase, Sensiscript™ Reverse Transcriptase, a hot-start *Taq* polymerase, RNase-free water, 0.6 µM of each primer and 6 U of RNase inhibitor (Roche Diagnostics, Laval, QC). Previously published primer sets (140-143) were used separately to amplify NoV, HAV and FCV (Table 3).

Amplifications were done in an Eppendorf Mastercycler gradient (Fisher Scientific) programmed specifically for each virus and primer set (Table 3). Positive and negative PCR controls were run simultaneously with each set of RT-PCR reactions. Positive controls included amplification of viral RNA extracted from cultured virus stocks (FCV, HAV), or from virus present in clinical stool specimens (NoV). Negative controls included 40 µl of PCR master mix and 10 µl of RNase-free H_2O, instead of the RNA template. RT-PCR amplicons were resolved by electrophoresis on 2% agarose gels at the rate of 135 volts for 45 min, followed by ethidium bromide staining and band detection using UV light.

2.9 Sequencing and Hybridizations

RT-PCR products of the expected band size were confirmed by Southern hybridization, dot blotting, or sequencing. Amplicons from outbreak samples were sent for sequencing to compare viral nucleotide sequences found in foods to those found in

clinical stool samples. For blotting experiments, specific probes (Table 3) were labeled at their 3' end with the DIG oligonucleotide 3' end labeling kit, 2nd generation (Roche Diagnostics), according to the manufacturer's instructions. Briefly, 100 pmoles of oligonucleotide were added to sterile 1.5 ml Eppendorf microcentrifuge tubes (VWR International) with 4 µl of 5 X reaction buffer, 4 µl $CoCl_2$, 1 µl DIG-ddUTP solution, 1 µl recombinant terminal transferase and DEPC-treated water (all reagents provided in the kit), for a total volume of 20 µl. Tubes were incubated in an Eppendorf thermomixer (VWR International) at 37°C for 15 min, then placed on ice. Lastly, 2 µl of 0.2 M EDTA (pH 8.0) were added to the samples to terminate the DIG-labeling reaction. Prepared probes were stored at -20°C until needed.

For dot blotting, 4 µl of each amplicon was denatured at 99°C/5 min, spotted onto an S&S Nytran® 0.2 µm nylon membrane (Schleicher & Schuell Bioscience Inc., USA) and the blotted DNA was cross-linked to the membrane by a 3-min exposure under 30 mJ UV light (GS Gene Linker™ UV chamber, BioRad Laboratories Ltd.).

For southern hybridizations, amplicons were transferred from 2% agarose gels to an S&S Nytran® 0.2 µm nylon membrane (Schleicher & Schuell Bioscience Inc.) using a 2 h TURBOBLOTTER™ Rapid Downward Transfer System (Schleicher & Schuell Bioscience Inc.), as per the manufacturer's instructions. Briefly, gels were initially incubated in denaturing buffer (3 M NaCl, 0.4 M NaOH) for 30 min, followed by the replacement of denaturing buffer for fresh solution and an additional 30 min incubation, to denature DNA amplicons. Gels containing denatured amplicons were then washed in transfer buffer (3 M NaCl, 8 mM NaOH) for 15 min and then set-up for transfer in the TURBOBLOTTER™ apparatus. Following transfer of the amplicons to membranes,

they were washed in neutralization buffer (0.2 M sodium phosphate, pH 6.8) and DNA was cross-linked to the membrane by exposure under 150 mJ UV light for 3 min (GS Gene Linker™ UV chamber, BioRad Laboratories Ltd.).

Subsequently, the membranes were pre-hybridized in DIG Easy Hyb (Roche Diagnostics) at 42°C for 30 min and then hybridized with the DIG-labeled probe at the same temperature for 2 h (dot blotting) or overnight (southern hybridization). Post-hybridization washes were also done at 42°C in low stringency buffer (2X SSC; SDS 0.1%) for 2 x 5 min, followed by washes in a high stringency buffer (0.5X SSC; SDS 0.1%) for 2 x 15 min. Hybridized 3'-end DIG-labeled probes were visualized by immunological color detection according to the instructions in the DIG Wash and Block Buffer Set (Roche Diagnostics) and the DIG Nucleic Acid Detection Kit (Roche Diagnostics).

Briefly, membranes were incubated in a series of buffers prior to color detection. Washes included: 1X washing buffer for 2 min, 1X blocking solution for 30 min, anti-DIG-alkaline phosphatase solution for 30 min, and 1X washing buffer for 2 x 15 min. Finally, membranes were equilibrated in 1X detection buffer for 5 min prior to adding color substrate solution (NBT/BCIP in detection buffer). Membranes were incubated in the dark from anywhere between a few minutes to 16 h. A positive result was identified as a blue precipitate on the membranes. Blue precipitate formation was due to an immunological reaction between the DIG-labeled RT-PCR-oligoprobe hybrids and an anti-digoxigenin alkaline phosphatase antibody conjugate via an enzyme-catalyzed colorimetric reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt substrates.

For sequencing, PCR amplicons were sent to DNA LandMarks (St-Jean-sur-Richelieu, QC) for complete analysis. Sequence analysis, using a modification of the Sanger dideoxy termination method, was done with an ABI 3700 capillary system.

Results

3.1 Viral Quantification

FCV, HAV, and NoV stocks were quantified by plaque assay and/or end point dilutions. Due to a lack of a cell culture system, end point dilution was used to quantify NoV. Briefly, 10-fold serial dilutions of a NoV RNA suspension were made in RNase-free water and 10 µl of each dilution was used in separate RT-PCR reactions. The highest dilution at which an amplicon (band) of expected size could be detected following gel electrophoresis was the end-point dilution. The viral titer was approximated by taking the reciprocal of that dilution and adjusting accordingly for the volume used in the RT-PCR (titer reported in RT-PCR units per ml). Using this method, an approximate viral titer was determined to be 10^8 RT-PCR units per ml. FCV and HAV were quantified by plaque assays (114, 137) and titers were 3×10^8 and 7×10^8 plaque forming units (PFUs)/ml, respectively. To compare viral quantification methods (plaque assay vs. end-point dilution), FCV and HAV titers were also measured by end-point dilutions and concentrations obtained were 10^7 and 10^8 RT-PCR units per ml, respectively. For HAV, titer remained the same with both methods of quantification while the FCV titer was one-log lower when using end-point dilutions for quantification (Table 4).

Table 4. Viral quantification by end point dilution and/or plaque assay. Ten-fold serial dilutions of each virus were prepared in EBSS and 100 µl of each dilution (ranging from 10^{-1} to 10^{-9}) were used to inoculate FRhK-4 (for HAV) or CrFK (for FCV) cell monolayers to determine the viral concentration (in plaque forming units per ml) by plaque assay. RT-PCR units per ml were determined by end point dilution where 140 µl of each dilution were taken and viral RNA was extracted with the QIAamp™ viral RNA mini-kit, then 10 µl of RNA were added to 40 µl of RT-PCR reaction mixture. Following RT-PCR and gel electrophoresis, the highest dilution at which a band of expected size could be observed was deemed the end point dilution and viral concentration was calculated by the reciprocal of this dilution. Each viral titer represents the average of three trials.

	End-point dilutions	Plaque assay
FCV	10^7 RT-PCR units/ml	3×10^8 PFUs/ml
HAV	10^8 RT-PCR units/ml	7×10^8 PFUs/ml
NoV	10^8 RT-PCR units/ml	NA[a]

[a] Not applicable. Unavailability of a cell culture system for the human noroviruses.

3.2 Comparison of RNA Extraction Methods

Various RNA extraction methods were compared to determine which would provide the greatest sensitivity when used in conjunction with the RT-PCR. Serial dilutions of FCV were made and RNA was extracted using each of three methods: heating 100 µl of virus at 95°C for 2 min followed by a 2-min incubation in a 50% ice bath, the QIAamp™ viral RNA mini-kit, and TRIzol® reagent. Following RT-PCR and visualization by gel electrophoresis, FCV detection limits were consistently the same for all three extraction methods (Table 5).

Table 5. Comparison of RT-PCR detection limits when using TRIzol® reagent, QIAamp™ Viral RNA Mini kit, or heat release (95°C, 2 min) to extract RNA from feline calicivirus particles. Extracted RNA was amplified for either 32 PCR cycles or 40 PCR cycles.

	Detection limits (dilutions)[a]	
	32 PCR cycles	40 PCR cycles
Heat release	10^{-3}	10^{-5}
QIAamp™ Viral RNA Mini-Kit	10^{-3}	10^{-5}
TRIzol® reagent	10^{-3}	10^{-5}

[a] Detection limits represent the average from three trials for each RNA extraction method.

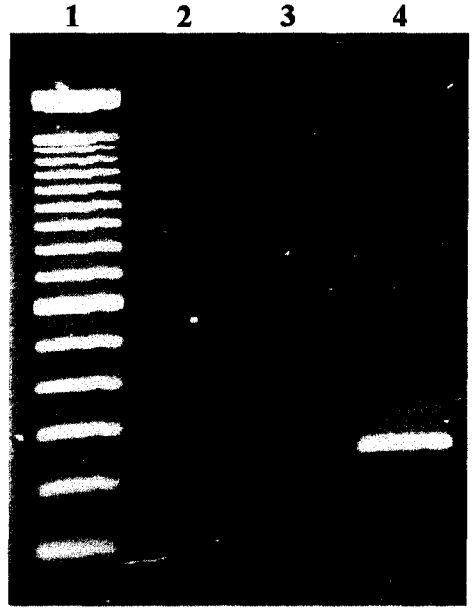
When extracting RNA from virus isolated from foods, however, the results were inconsistent when using different RNA extraction methods. In later studies, lettuce samples were inoculated with serial dilutions of HAV, concentrated with the Pathatrix™ system and RNA was extracted by heating the isolated virus (95°C for 2 min) or by using the QIAamp™ viral RNA mini-kit. Amplicons of the expected size were consistently detected when using the QIAamp™ viral RNA mini-kit, while bands were not always detected when using heat to extract RNA from isolated virus particles (Figure 6). Consequently, the QIAamp™ viral RNA mini-kit was used in all subsequent studies for its consistency in viral yield, enhanced sensitivity when amplified in the RT-PCR, and ease of use.

3.3 Method 1 Development - Sample Processing with TRIzol® Reagent

The objective of this study was to develop a rapid and user-friendly viral detection method for ready-to-eat foods. Reports of viral transmission via sandwiches have been well documented so that food commodities chosen for these studies included, but were not limited to, deli-sliced ham and white bread. Kingsley *et al.* (100) previously published a method in which TRIzol® reagent was used to concentrate norovirus and hepatitis A virus particles from oysters. In initial studies, a similar protocol was used to assess the efficiency at which TRIzol® reagent could isolate viral RNA from other food commodities. Feline calicivirus was used as a surrogate agent for noroviruses in all method development experiments with TRIzol® reagent.

Initially, 20 g of sliced deli-ham was inoculated with 10^5, 10^4, and 10^3 PFUs of FCV and processed with the TRIzol® reagent, with resulting extracts containing total RNA. Isolated RNA pellets were resuspended in RNase-free water and amplified by the

Figure 6. RNA extraction using the QIAamp™ Viral RNA Mini-Kit vs. heat release of RNA from capsid. Twenty-five grams of lettuce was inoculated with 10^6 PFUs of hepatitis A virus and processed in the Pathatrix™. RNA was released from half of the captured virus particles using the QIAamp™ Viral RNA Mini-Kit and the other half with heat (95°C, 2 min). Following RT-PCR and gel electrophoresis, amplicons of expected size (218 bp) were detected. Lane 1: 100 bp DNA ladder, Lane 2: RT-PCR negative control, Lane 3: RNA released with heat, Lane 4: QIAamp™ Viral RNA Mini Kit.


1
2
3
4

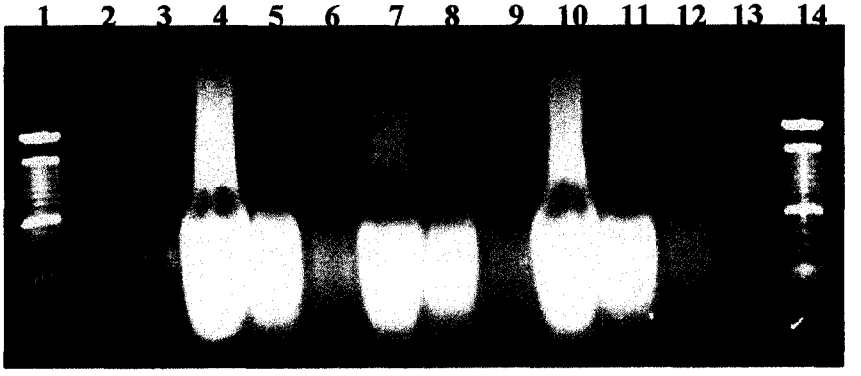
RT-PCR prior to detection. The co-extraction of extraneous material from foods was evident, as seen by the turbidity in the final RNA suspensions and/or the difficulty in dissolving the RNA pellets. Following gel electrophoresis, amplicons (bands) of the expected size could not be detected, but streaking was apparent in each loaded lane (Figure 7). Streaking was still observed after diluting each RNA suspension 10- and 100-fold prior to the RT-PCR (Figure 7). Visualization of discrete bands was expected given the high titer of virus used (10^5 PFU) to inoculate the ham. The turbidity of the suspensions and streaking patterns suggested that RNA extracts contained residual compounds that interfered with the activity of enzymes used in the PCR, leading to false-negative results and extensive background noise.

3.3.1 Purification of RNA Suspensions Following TRIzol® Processing

To remove residual substances, nucleic acid purification methods were applied to RNA suspensions isolated from food samples. Three commercially-available products were compared for their effectiveness at removing these materials from RNA isolates.

Product 1: 30 µl of RNA suspension was added to the InstaGene™ Matrix and mixed for 30 min at 56°C with an Eppendorf thermomixer set to that temperature, followed by 10 min at 99°C. During this time, the matrix absorbed cell lysis products which, following a 4 min centrifugation step (10,000 x g) sedimented to the bottom of a 1.5 ml centrifugation tube with RNA remaining in the upper aqueous layer. However, residual compounds (i.e., non specific RNA, excess primers) were not entirely removed from the RNA extracts, as shown by streaking patterns still observed on gels following DNA amplification. PCR products of the expected band size could only be detected if

Figure 7. Concentration and detection of feline calicivirus (FCV) from artificially-inoculated ham with TRIzol® reagent and RT-PCR. FCV-inoculated ham samples were processed with TRIzol® reagent, according to the manufacturer's instructions. Amplicons of expected band size (218 bp) were detected following electrophoresis on a 2% agarose gel. Lane 1: 100 bp DNA ladder, Lane 2: RT-PCR negative control, Lane 3: RT-PCR positive control, Lane 4: 10^3 PFU/20g ham, Lane 5: 10^3 PFU/20g ham with 10-fold dilution of RNA, Lane 6: 10^3 PFU/20g ham with 100-fold dilution of RNA, Lane 7: 10^2 PFU/20g ham, Lane 8: 10^2 PFU/20g ham with 10-fold dilution of RNA, Lane 9: 10^2 PFU/20g ham with 100-fold dilution of RNA, Lane 10: negative control (water)/20g ham, Lane 11: negative control with 10-fold dilution, Lane 12: negative control with 100-fold dilution, Lane 13: empty, Lane 14: 100 bp DNA ladder.


1 2 3 4 5 6 7 8 9 10 11 12 13 14

the InstaGene™ Matrix-treated RNA suspensions were diluted 100-fold prior to the RT-PCR (Figure 8).

Product 2: The QIAshredder™ homogenizer is a mini-spin column designed to homogenize RNA pellets to yield uniform RNA suspensions. Theoretically, this process filters out insoluble debris to reduce RNA extract viscosity. RNA suspensions were added to a spin column, centrifuged (10,000 x g) for 2 min, and filtered through the column into a 1.5 ml tube. Following RT-PCR amplification, amplicons were not detected following the use of this column (Figure 9).

Product 3: FCV genomic RNA was isolated from total RNA suspensions with Dynabeads® Oligo $d(T)_{25}$. Magnetic beads are bound to oligo d(T) sequences designed to anneal to poly A tails on genomic or messenger RNA. Once bound to the oligo d(T), polyadenylated RNA (such as FCV genomic RNA) is magnetically separated (using a magnetic bead attractor) from residual food compounds and other RNA molecules present in the suspension. Clear bands of the expected size were detected when FCV-inoculated deli-ham samples were processed with TRIzol® and RNA extracts purified with Oligo $d(T)_{25}$ beads (Figure 10A). Bands of the expected size were visible without the need for RNA dilution, suggesting that the contaminating materials were effectively separated from the genomic RNA.

3.3.2 Modification of TRIzol® Methodology for Applicability to Breads

The TRIzol® method described above was not very successful at detecting virus in inoculated bread samples. This is because when 5 g of FCV-inoculated white bread was washed with TRIzol®, the food matrix completely absorbed the TRIzol® so that virus-containing wash could not be recovered. Therefore, a slight modification was

Figure 8. Concentration and detection of feline calicivirus (FCV) from artificially-inoculated ham with TRIzol® reagent, InstaGene™ Matrix and RT-PCR. FCV-inoculated ham samples were processed with TRIzol® reagent, and the resuspended RNA pellet was purified with InstaGene™ Matrix. Both procedures were done according to the manufacturer's instructions. Amplicons of expected band size (218 bp) were detected following electrophoresis on a 2% agarose gel. Lane 1: 100 bp DNA ladder, Lane 2: RT-PCR negative control, Lane 3: RT-PCR positive control, Lane 4: 10^5 PFU/20g ham, Lane 5: 10^5 PFU/20g ham with 10-fold dilution of RNA, Lane 6: 10^5 PFU/20g ham with 100-fold dilution of RNA, Lane 7: 10^4 PFU/20g ham, Lane 8: 10^4 PFU/20g ham with 10-fold dilution of RNA, Lane 9: 10^4 PFU/20g ham with 100-fold dilution of RNA, Lane 10: 10^3 PFU/20g ham, Lane 11: 10^3 PFU/20g ham with 10-fold dilution of RNA, Lane 12: 10^3 PFU/20g ham with 100-fold dilution of RNA.

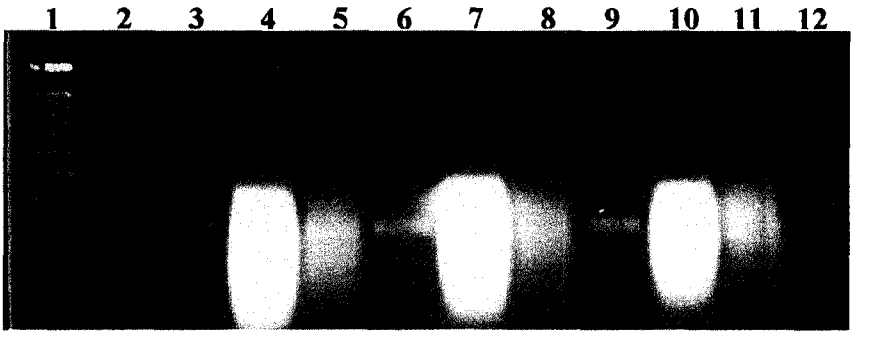

1 2 3 4 5 6 7 8 9 10 11 12

Figure 9. Concentration and detection of feline calicivirus (FCV) from artificially-inoculated ham with TRIzol® reagent, QIAshredder™ homogenizer and RT-PCR. FCV-inoculated ham samples were processed with TRIzol® reagent and the resulting pellet was resuspended and homogenized in a QIAshredder™ homogenizer spin column. Both procedures were done according to the manufacturer's instructions. Amplicons of expected band size (218 bp) were detected following electrophoresis on a 2% agarose gel. Lane 1: 100 bp DNA ladder, Lane 2: RT-PCR negative control, Lane 3: RT-PCR positive control, Lane 4: 10^4 PFU/20g ham, Lane 5: 10^4 PFU/20g ham with 10-fold dilution of RNA, Lane 6: 10^4 PFU/20g ham with 100-fold dilution of RNA, Lane 7: 10^3 PFU/20g ham, Lane 8: 10^3 PFU/20g ham with 10-fold dilution of RNA, Lane 9: 10^3 PFU/20g ham with 100-fold dilution of RNA, Lane 10: negative control (water)/20g ham, Lane 11: negative control with 10-fold dilution, Lane 12: negative control with 100-fold dilution.

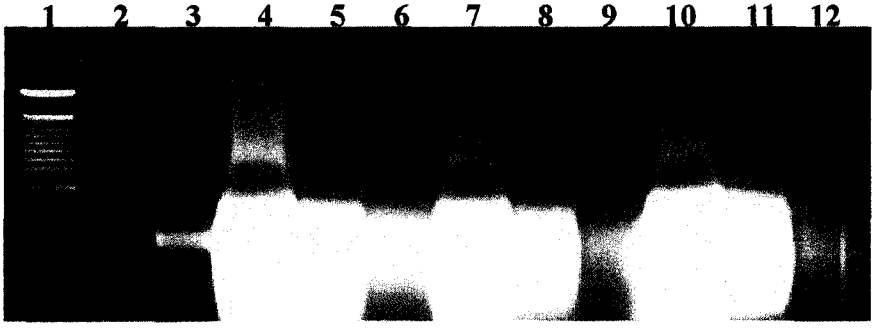

1 2 3 4 5 6 7 8 9 10 11 12

Figure 10. Detection limits for the concentration and detection of feline calicivirus from various foodstuff using TRIzol® and Dynabeads® Oligo $d(T)_{25}$.

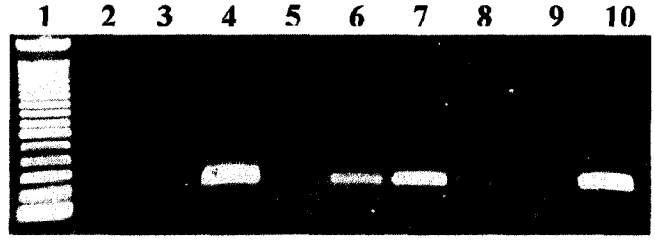
FCV-inoculated foods were treated with TRIzol® reagent and the extracted RNA was magnetically separated from other nucleic acids and food residues with Oligo $d(T)_{25}$ beads. In A), half of the RNA suspension (bound to Oligo $d(T)_{25}$ beads) was heated to 90°C for 2 min to detach the beads and RT-PCR was performed in parallel for RNA samples with and without beads attached. Amplicons of expected band size (218 bp) were detected following electrophoresis on a 2% agarose gel and detection limits were determined.

A) **FCV-inoculated ham**. Lane 1: 100 bp DNA ladder, Lane 2: RT-PCR negative control, Lane 3: empty, Lane 4: RT-PCR positive control, Lane 5: 10^3 PFU/20g ham with beads in PCR, Lane 6: 10^4 PFU/20g ham with beads in PCR, Lane 7: 10^5 PFU/20g ham with beads in PCR, Lane 8: 10^3 PFU/20g ham without beads in PCR, Lane 9: 10^4 PFU/20g ham without beads in PCR, Lane 10: 10^5 PFU/20g ham without beads in PCR.

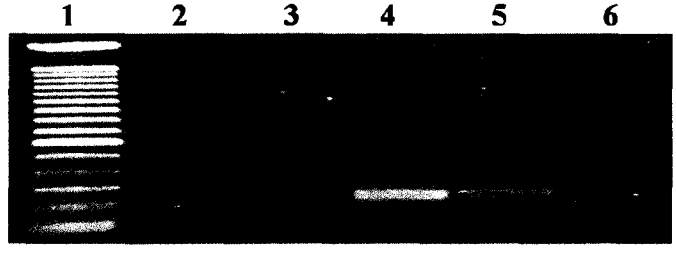
B) **FCV-inoculated bread.** Lane 1: 100 bp DNA ladder, Lane 2: RT-PCR negative control, Lane 3: RT-PCR positive control, Lane 4: 10^5 PFU/5g bread, Lane 5: 10^4 PFU/5g bread, Lane 6: 10^3 PFU/5g bread.

C) **FCV-inoculated lettuce.** Lane 1: 100 bp DNA ladder, Lane 2: RT-PCR negative control, Lane 3: RT-PCR positive control, Lane 4: 10^6 PFU/20g lettuce, Lane 5: 10^5 PFU/20g lettuce, Lane 6: negative control (water)/20g lettuce.

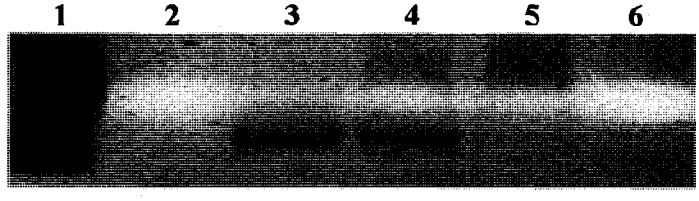
A. Ham



B. Bread



C. Lettuce

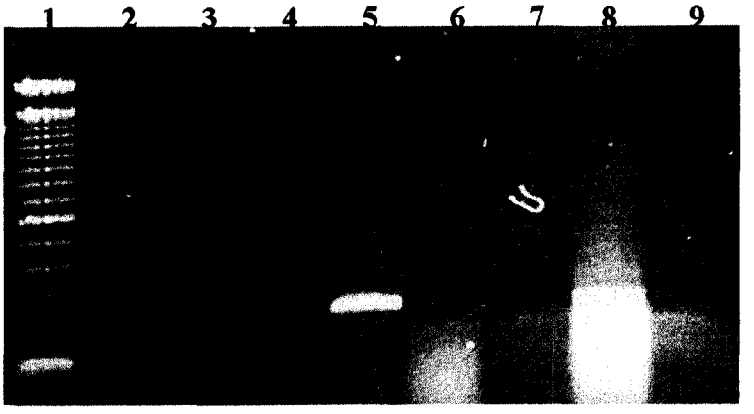


introduced to adapt this method to detection in bread. As such, an initial elution step was added to allow detachment of the virus particles from the bread surface. To facilitate this process, the samples were emulsified by vortexing. The resulting virus-containing wash was processed with the TRIzol® methodology described for ham and the resulting RNA suspension was purified with Oligo $d(T)_{25}$ beads. Various elution buffers were compared for their efficacy at separating the virus from bread surfaces; these included 15 ml of either distilled water, 0.9% NaCl solution, or glycine buffer (0.1 M glycine, 0.3 M NaCl, pH 9.5). A band of the expected size was clearly detected following gel electrophoresis when using glycine buffer to saturate the bread in an initial saturation step (Figure 11). This additional elution step lengthens the procedure and was only necessary with breads or foods of similar composition, as the goal was to produce a virus-containing eluant prior to processing with the TRIzol® procedure.

3.4 Detection Limits in Select Foods Following TRIzol® Processing and Oligo $d(T)_{25}$ Purification

To determine the applicability of the method on components of a whole sandwich, deli-sliced ham, white bread, and iceberg lettuce were each inoculated with serial dilutions of FCV (ranging from 10^5 PFU to 10^3 PFU) and processed with the TRIzol® reagent and Oligo $d(T)_{25}$ beads. Virus detection limits (i.e., sensitivity of detection) were determined for each food item based on RT-PCR analysis of RNA extracts. To be detected, at least 10^3 PFUs per 20 g ham, 10^4 PFUs per 5 g of bread, and 10^6 PFUs per 20 g of lettuce had to be artificially-inoculated onto food samples. Virus could not be detected in foods inoculated with lower virus concentrations than the limits mentioned herein (Figure 10).

Figure 11. Comparison of various buffers for the elution of FCV from inoculated breads prior to processing with TRIzol® reagent. Bread samples inoculated with 10^6 PFUs of FCV were initially saturated with 15 ml of either distilled water, 0.9% NaCl, or glycine buffer (0.1 M glycine, 0.3 M NaCl, pH 9.5). The resulting wash was then processed with TRIzol® reagent and 1 µl of RNA was added to 24 µl of RT-PCR reaction mixture and amplified for 32 cycles. Amplicons of expected band size (218 bp) were detected following electrophoresis on a 2% agarose gel. Lane 1: 100 bp DNA ladder, Lane 2: empty, Lane 3: RT-PCR negative control, Lane 4: empty, Lane 5: RT-PCR positive control, Lane 6: initial saturation with double distilled water, Lane 7: initial saturation with 0.9% NaCl, Lane 8: initial saturation with glycine buffer, Lane 9: initial saturation with 0.9% NaCl.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3.5 Processing of Outbreak Food Samples with TRIzol® Reagent

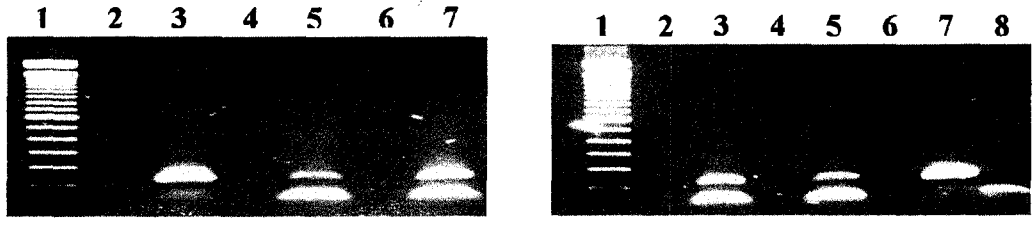
Suspected food items implicated in two separate NoV outbreaks (Ottawa, ON and Guelph, ON) were received in March 2004 and processed with TRIzol®. Samples were received prior to the incorporation of Dynabeads® Oligo $d(T)_{25}$ beads as an RNA purification step. Following TRIzol® washes, isolated RNA was treated with InstaGene™ Matrix and 10- and 100-fold dilutions were made prior to the RT-PCR. NoV was detected in spinach salad, beef salad, pasta salad and salami (4 out of 16 samples processed) (Figure 12). Amplicons were confirmed by Southern blotting for the beef salad and pasta salad (Figure 12). Certain food items such as creamy macaroni salad, cakes, muffins and salad dressings could not be processed due to their thick or spongy consistencies. Modifications made to the method since then would have probably allowed more samples to be tested (i.e., saturation with a buffer prior to viral extraction with TRIzol®).

3.6 Method 2 Development – Sample Processing with the Pathatrix™

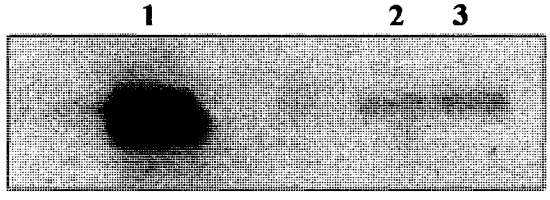
The TRIzol® methodology was not sufficiently sensitive to detect the low levels of virus usually found in foods. Therefore, the Pathatrix™ system, which has successfully been applied to the concentration and detection of bacteria from various foods, was tested for its ability to concentrate viruses from select food matrices. This system consists of a motorized equipment that houses five independent processing units, each of which has its own pump head and a control unit for the temperature, flow rate, and processing time. For processing in this system, each food sample was placed in a stomacher bag containing an elution buffer and positively charged (cationic) magnetic beads. The beads were forcefully circulated throughout the food sample allowing them

Figure 12. Processing of various norovirus outbreak food samples with TRIzol® reagent and InstaGene™ Matrix.
Food samples from norovirus outbreaks in Ottawa, ON and Guelph, ON were processed with TRIzol® reagent and the resuspended pellet was treated with InstaGene™ Matrix. Cleaned RNA was amplified by RT-PCR and detected by gel electrophoresis (123 bp amplicons). A) Left gel. Lane 1: 100 bp DNA ladder, Lane 3: RT-PCR positive control, Lane 5: spinach salad, Lane 7: penne salad. Right gel. Lane 1: 100 bp DNA ladder, Lane 3: beef salad, Lane 5: cold cuts, Lane 7: RT-PCR positive control, Lane 8: RT-PCR negative control. B) Southern hybridization. 1: RT-PCR positive control, 2: penne salad, 3: beef salad.

A)



B)



to interact with the virus. In early experiments, HAV-inoculated lettuce and strawberries and NoV-inoculated ham were homogenized prior to processing in the Pathatrix™. In all cases, detection limits were less sensitive than when solid foods were processed (Table 6). Homogenized food sample suspensions became viscous when puréed and significantly fewer beads were visible on the capture magnet of the Pathatrix™ equipment, suggesting that the beads were trapped within the thick food particle suspension.

Table 6. Homogenization of select HAV or NoV-inoculated food samples prior to processing in the Pathatrix™ system.

Food	Virus	Detection Limits (PFUs/25g)	
		Homogenized	Non-homogenized
Lettuce	HAV	10^7	10^2
Strawberries		10^5	10^4
Ham	NoV	10^7	10^4

Food items were suspended in either EBSS (pH 7.2) or glycine buffer (pH 8.5) prior to processing in the Pathatrix™ machinery. When adding strawberries to EBSS (pH 7.2), the acidity of the berries reduced the pH of the buffer to approximately 6.8. Virus recovery, however, was improved when 0.1 N NaOH was added to the stomacher bag to readjust the pH to 7.2 (Table 7).

Table 7. Enhancement of virus capture sensitivity from select foods with different elution buffers. Detection limits were compared when adding HAV- or NoV-spiked foods to either EBSS or glycine buffer and circulating 100 µl of cationic beads throughout the samples for 30 min in the Pathatrix™ machinery. RNA was extracted from the captured virus with the QIAamp™ viral RNA mini-kit, followed by RT-PCR and gel electrophoresis. Each experiment was done in triplicate.

Food	Virus	Detection Limits		
		EBSS (pH 6.8)[a]	EBSS (pH 7.2)	Glycine buffer (pH 8.5)
Strawberries	HAV	10^5	$10^{4\,b}$	10^{-1}
Green onions		NA[c]	10^5	10^0
Lettuce	NoV	NA	10^4	10^2
Ham		NA	10^5	10^6

[a] Final pH of the buffer after adding the strawberries.
[b] Once strawberries were added to the buffer, pH was adjusted to 7.2 with 1 N NaOH.
[c] Not applicable. No trials were done in these conditions.

Virus recovery was at least 100-fold greater when using glycine buffer (pH 8.5) over EBSS (pH 7.2) for elution of viruses from lettuce, strawberry and green onion surfaces (Table 8). Interestingly, NoV recovery was 10-fold greater when using EBSS (pH 7.2) over glycine buffer (pH 8.5) to elute virus from ham (Table 7).

Vials containing the cationic beads were briefly vortexed and either 50, 100, 200 or 300 µl were added to stomacher bags prior to food processing in the Pathatrix™ machinery, to determine which amount provided the most sensitive results. A 100-fold increase in sensitivity could be observed when 100 µl vs. 50 µl of beads was added to HAV-inoculated lettuce samples. No differences in sensitivity were observed when increasing the volume of beads from 100 to 200 µl (Table 8).

Table 8. Enhancement of virus capture from select foods with different volumes of cationic beads. Virus-inoculated foods were processed in the Pathatrix™ system for 30 min with varying amounts of cationic beads. Detection limits were compared following RNA extraction of the isolated virus with the QIAamp™ viral RNA mini-kit, RT-PCR and gel electrophoresis. Each experiment was done in triplicate.

Food	Virus	Buffer	Detection limits (PFUs/25g)			
			50 ul	100 ul	200 ul	300ul
Lettuce	HAV	Glycine buffer (pH 8.5)	10^2	10^0	10^0	NA[a]
Strawberries	NoV	EBSS (pH 7.2)	NA	10^6	10^4	10^4

[a] Not applicable. No trials were done in these conditions.

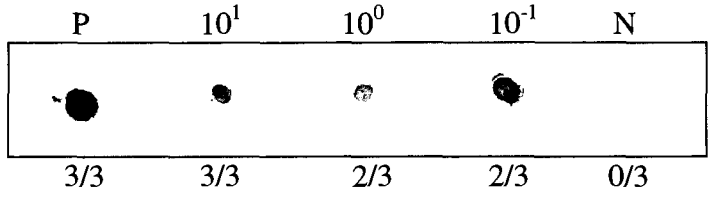
Similar trends were observed in HAV-inoculated strawberries when increasing the amount of beads from 100 to 200 µl, with no increase in sensitivity at 300 µl of beads (Table 8).

3.7 Detection Limits in Select Foods Following Pathatrix™ Processing

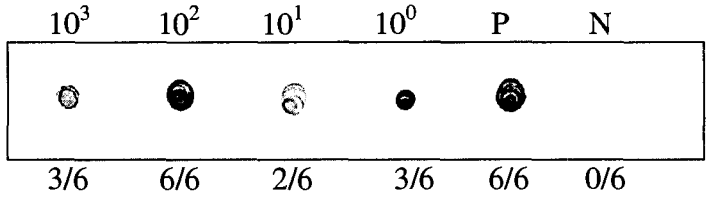
The optimized method for virus capture from select foods included processing samples in the Pathatrix™ system with glycine buffer (pH 8.5) and 100 µl of cationic beads, and extracting RNA with the QIAamp™ Viral RNA Mini-Kit. Using these parameters, the detection limits in lettuce, strawberries and green onions were 1, 0.1, and 1 PFUs of HAV, respectively (Figure 13). For noroviruses, capture in the Pathatrix™ was less effective and the virus could only be detected when food samples were initially inoculated with at least 10^2, 10^4, and 10^5 RT-PCR units per 25 g of lettuce, ham and strawberries, respectively (Figure 14). During detection limit studies, virus could not always be recovered and detected from foods inoculated with high virus concentrations while, in the same trial, virus could be detected in foods spiked with lower inocula of virus (Figure 15).

Figure 13. Detection limits for the concentration and detection of hepatitis A virus from various foodstuff using the Pathatrix™ system.
HAV-inoculated foods were processed for 30 min in the Pathatrix™ system with 100 µl of cationic beads and 225 ml of glycine buffer (0.5 M glycine, 0.14 M NaCl, 0.2% Tween 20, pH 8.5). The isolated virus was washed with PBS and viral RNA was extracted with the QIAamp™ viral RNA mini-kit, according to the manufacturer's instructions. Amplicons of expected band size (247 bp) were confirmed by dot blotting and detection limits were determined. The notations above the figures indicate the initial inocula of virus (PFU per 25 g of food). Positive and negative controls are denoted as P and N, respectively, where negative controls included the processing of foods that were not inoculated with virus, while positive controls consisted of HAV RNA added to the RT-PCR reaction mix. Notations below the figures indicate the number of times an amplicon was detected at each inoculum level.

A. Strawberries



B. Lettuce



C. Green onions

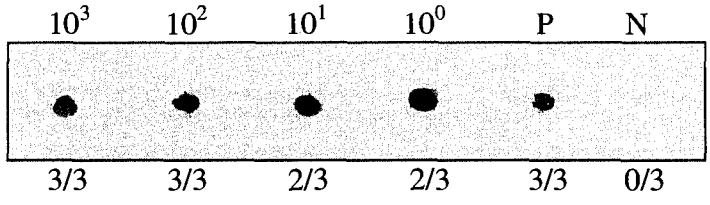
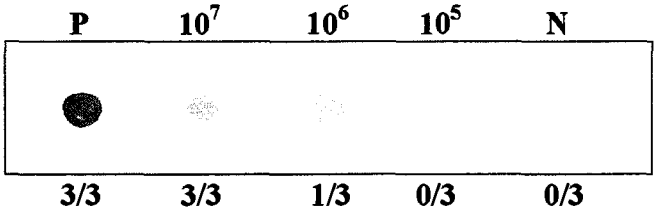
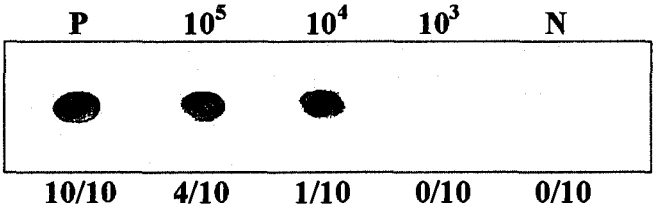


Figure 14. Detection limits for the concentration and detection of norovirus from various foodstuff using the Pathatrix™ system.
NoV-inoculated foods were processed for 30 min in the Pathatrix™ system with 100 µl of cationic beads and 225 ml of glycine buffer (0.5 M glycine, 0.14 M NaCl, 0.2% Tween 20, pH 8.5). The isolated virus was washed with PBS and viral RNA was extracted with the QIAamp™ viral RNA mini-kit, according to the manufacturer's instructions. Amplicons of expected band size (398 bp) were confirmed by dot blotting and detection limits were determined. The notations above the figures indicate the initial inocula of virus (PFU per 25 g of food). Positive and negative controls are denoted as P and N, respectively, where negative controls included the processing of foods that were not inoculated with virus, while positive controls consisted of NoV RNA added to the RT-PCR reaction mix. Notations below the figures indicate the number of times an amplicon was detected at each inoculum level.

A. Strawberries



B. Ham



C. Lettuce

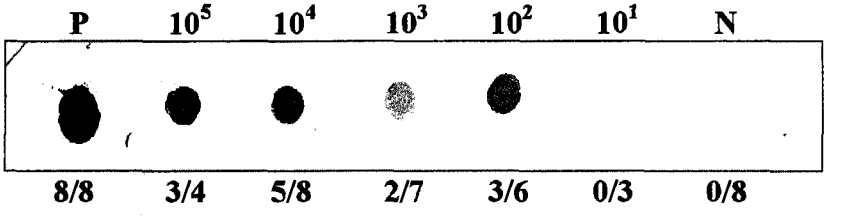
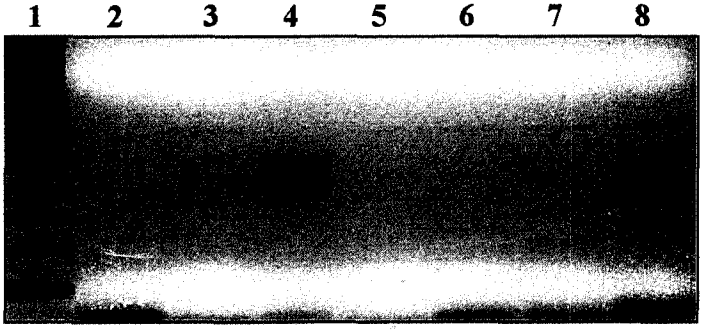


Figure 15. Detection limits for the concentration and detection of norovirus from lettuce samples using the Pathatrix™ system.
Twenty-five gram samples of iceberg lettuce were inoculated with serial ten-fold dilutions of norovirus and processed for 30 min in the Pathatrix™ system with 100 µl of cationic beads and 225 ml of glycine buffer (0.5 M glycine, 0.14 M NaCl, 0.2% Tween 20, pH 8.5). The isolated virus was washed with PBS and viral RNA was extracted with the QIAamp™ viral RNA mini-kit, according to the manufacturer's instructions. Following RT-PCR and gel electrophoresis, amplicons of expected size (398 bp) were detected when foods were initially inoculated with lower virus concentrations (lanes 7,8) while virus was undetectable when foods were initially spiked with higher viral concentrations (lanes 5,6). Lane 1: 100 bp DNA ladder, Lane 2: RT-PCR negative control, Lane 3: negative control (water)/25g lettuce, Lane 4: RT-PCR positive control, Lane 5: 10^5 RT-PCR units/25g lettuce, Lane 6: 10^4 RT-PCR units/25g lettuce, Lane 7: 10^3 RT-PCR units/25g lettuce, Lane 8: 10^2 RT-PCR units/25g lettuce.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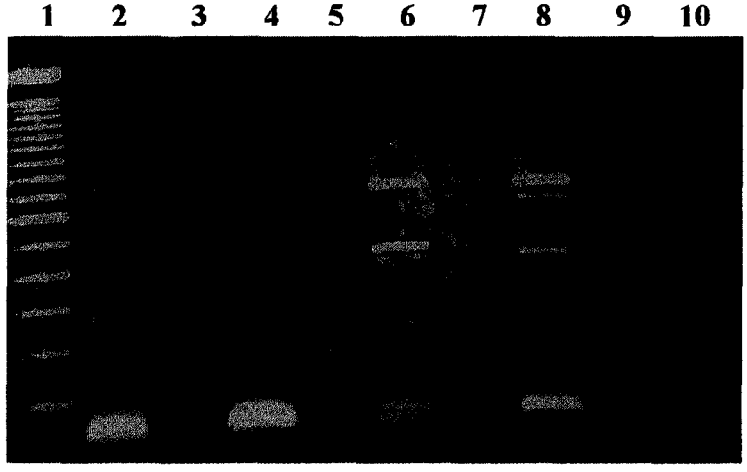
3.8 Processing of Outbreak Food Samples with the Pathatrix™ System

Food samples involved in a norovirus outbreak in Prince Edward Island (July 2005) were processed with the developed Pathatrix™ methodology. Briefly, 200 µl of cationic beads were added to each stomacher bag and re-circulated within food samples for 1 h at room temperature. RNA was extracted from isolated virus particles with the QIAamp™ viral RNA mini-kit and 10 µl of each RNA suspension was used for amplification in the RT-PCR prior to detection. Noroviruses were detected in potato salad, iceberg lettuce, diced tomatoes and mixed coleslaw (4/10 samples processed) (Figure 16). The viral strain isolated from foods was identical to the one found in stools of an infected individual. BLAST (www.pubmed.com; select genome) results indicated that the isolated strain was a GIIb norovirus, with 97-98% identity to the Australian Hu/Sydney/C14/2002/AU recombinant virus (Genbank accession number AY845056) (30). Mayonnaise was one of the food samples received, but it could not be processed due to its thick and sticky consistency. As such, no beads were recovered on the magnet following processing in the Pathatrix™.

3.9 Competition Studies

Magnetic beads used in these studies to capture viruses in the Pathatrix™ system are positively charged and non-specific. In principle, any microorganism or molecule carrying a negative charge could interact with these beads so that they most likely bind and capture numerous negatively charged entities during Pathatrix™ processing. To determine whether the presence of multiple contaminants in a food sample would affect the ability of the cationic beads to capture the pathogen of interest, two viruses were inoculated onto each food item. Twenty-five gram samples of iceberg lettuce were

Figure 16. Processing of various norovirus outbreak food samples with the Pathatrix™ system.
Various food samples implicated in a norovirus outbreak in Darnley, P.E.I. (summer 2005) were processed with the Pathatrix™ system in conjunction with positively charged magnetic beads. RNA from the captured virus was extracted with the QIAamp™ viral RNA mini-kit and 10 µl was used for RT-PCR. Following gel electrophoresis, bands of expected size (398 bp) were excised from the gel and sent for sequencing. Lane 1: 100 bp DNA ladder, Lane 2: carrots, Lane 3: empty, Lane 4: potato salad, Lane 5: empty, Lane 6: iceberg lettuce, Lane 7: empty, Lane 8: diced tomatoes, Lane 9: empty, Lane 10: mixed coleslaw. Carrots did not contain any detectable virus, as shown in lane 2.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simultaneously inoculated with 10^3, 10^2, 10^1, or 10^0 PFUs of HAV and 10^6 PFUs of FCV. In order to stay consistent with the procedure established in section 3.6, glycine buffer (pH 8.5) and 200 µl of cationic beads were added to the samples and processed in the Pathatrix™ for 30 min. Following RNA extraction and RT-PCR amplification, amplicons of expected band size were detected up to an initial inoculum of 10^0 PFU of HAV (Figure 17A). FCV was also detected in all food samples as shown in Figure 17B. These results indicate that capture sensitivity was not affected by the presence of a second contaminant in the food sample.

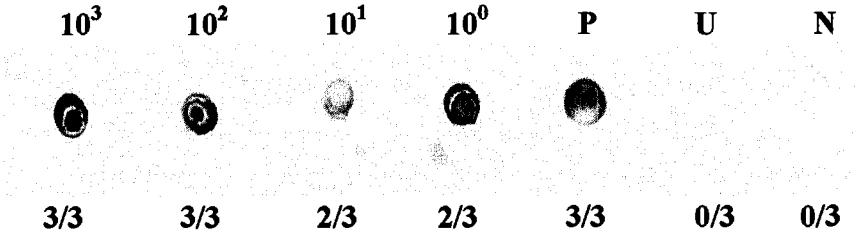
Figure 17. Bead competition studies with foods simultaneously inoculated with HAV and FCV.

Twenty-five gram samples of lettuce were simultaneously inoculated with 10^6 PFUs of FCV and dilutions of HAV (ranging from 10^3 to 10^0) and processed in the Pathatrix™ system with 200 µl of cationic beads. Captured virus was extracted with the QIAamp™ viral RNA mini-kit and 10 µl each of RNA was added to RT-PCR reaction mixtures containing either FCV- or HAV-specific primers. Following separate thermocycling programs, amplicons were either detected following dot blotting (A. HAV) or gel electrophoresis (B. FCV), with an expected band size of 218 bp.

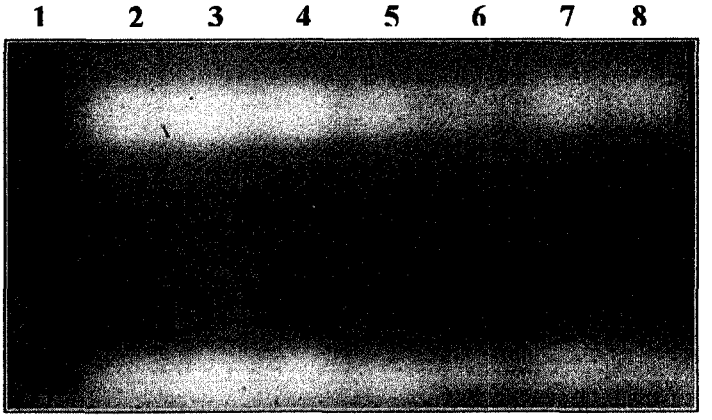
A) **HAV detection.** The notations above the figures indicate the initial inocula of HAV (PFU per 25 g of lettuce). Positive and negative controls are denoted as P, U, and N, where negative controls (U, N) included the processing of foods that were not inoculated with virus or RT-PCR reactions that did not contain RNA, while positive controls consisted of HAV RNA added to the RT-PCR reaction mix. Notations below the figures indicate the number of times an amplicon was detected at each inoculum level.

B) **FCV detection.** Lane 1: 100 bp DNA ladder, Lane 2: RT-PCR negative control, Lane 3: negative control (water)/25g lettuce, Lane 4: FCV RT-PCR positive control, Lane 5: 10^6 PFUs FCV + 10^3 PFUs HAV/25g lettuce, Lane 6: 10^6 PFUs FCV + 10^2 PFUs HAV/25g lettuce, Lane 7: 10^6 PFUs FCV + 10^1 PFUs HAV/25g lettuce, Lane 8: 10^6 PFUs FCV + 10^0 PFUs HAV/25g lettuce

A.



B.



Discussion

Rapid and sensitive methods for the detection of viruses from foods are needed during outbreak situations, but their development has been an ongoing challenge for food microbiologists. Many of the commonly used detection methods, such as the plaque assay, are not applicable to the most important foodborne viruses, because these viruses have no established cell culture system (NoV) or propagate very poorly and slowly in the cell culture systems developed (HAV). Moreover, many of the existing detection techniques, such as electron microscopy, require a very large amount of virus for detection (at least 10^6-10^7 particles/ml of specimen) (144) so that RT-PCR is most often the detection method of choice. Although highly sensitive, the RT-PCR is only successful at detecting viruses when these have been efficiently isolated from foods, with minimal co-extraction of inhibitory molecules. Such molecules have often been reported in final RNA preparations and greatly reduced the detection sensitivity, thus necessitating that additional steps be added to the methods in order to remove these inhibitory contaminants (102, 103, 113, 118). The need for further processing takes more time and reagents, and can potentially cause loss of some RNA.

The inhibitory effect of residual food molecules was observed during initial studies where FCV was concentrated from spiked deli-ham using TRIzol® reagent. Although the TRIzol® reagent provided for a viral isolation method which was simple and straightforward, co-extracted molecules present in the RNA suspensions (as seen with the observed turbidity of final extracts) were a major drawback to this method. Without purification of the isolated RNA, patterns of streaking were observed following RT-PCR and gel electrophoresis, indicating non-specific amplification and leading to

false-negative results (Figure 7). Despite diluting RNA suspensions prior to RT-PCR, clearly defined bands were still unapparent following gel electrophoresis. The TRIzol® reagent has been used by other researchers to elute noroviruses and/or hepatitis A viruses from shellfish (100) or delicatessen meats such as turkey, ham and roast beef (107). Our data confirm previous reports by Schwab *et al.* (107) in that the presence of co-extracted inhibitors affected amplification of viral RNA, so that 100-fold dilutions of RNA isolates and a nested-PCR approach were necessary for viral detection.

Other complications with the use of TRIzol® reagent include its inapplicability to complex food matrices such as breads, cakes, and matrices of thick consistency (i.e., salad dressings). When attempting to process breads with this product, TRIzol® was completely absorbed so that no eluant could be obtained prior to processing with bromochloropropane and isopropanol. To address this problem, an initial bread saturation step with glycine buffer was added to the protocol in an attempt to elute the virus from the food surface and process the resulting solution with TRIzol® reagent. Though this initial saturation step provided an eluant to which TRIzol® could then be added, the virus could still not be detected after RT-PCR (data not shown). The lack of detectable amplicons could be due to several factors such as: i) the elution buffer used was inefficient at separating the virus from the bread surface; ii) the virus was lost by denaturation in subsequent processing steps; or iii) PCR inhibitors were co-extracted with the virus and lead to false-negative results.

We decided to explore this last possibility since PCR inhibition had been the reason for the poor detection limits obtained in ham. To remove inhibitors from shellfish samples, Kingsley *et al.* (100) used an approach where TRIzol®-isolated viral RNA was

successfully purified with oligo (dT)-labeled magnetic beads. Similarly, we compared various RNA purification methods for their ability to enhance detection sensitivity. Attempts at removing inhibitors from RNA suspensions were done with InstaGene ™ matrix, QIAshredder™ homogenizer, or Oligo $(dT)_{25}$ beads. InstaGene™ matrix was the first product used to clarify RNA suspensions and it was unsuccessful at removing excessive extraneous material from samples. Thus, the purified RNA extracts still needed to be diluted 100-fold prior to RT-PCR (Figure 8). Furthermore, the relatively low sensitivity of detection obtained (10^4 PFU FCV per 20 g ham) indicated that although this product was designed to absorb cell lysis products interfering with PCR, it did not efficiently absorb residual food particles present in RNA suspensions.

The QIAshredder™ homogenizer is a shredding system consisting of a biopolymer microcentrifuge spin column and is designed to homogenize tissue lysates and to filter out insoluble high molecular weight molecules in order to reduce sample viscosity. Although ineffective in our studies, Sair *et al.* (111) reported that a combination of TRIzol® for RNA extraction followed by the QIAshredder™ homogenizer for RNA resolubilization, yielded the best RT-PCR detection limits when compared to other treatments. They reported detection limits at initial inocula of <10 and <100 PFU of HAV per 6 g of hamburger and lettuce, respectively, and <5 and <50 RT-PCR units of NoV per 6 g of hamburger and lettuce, respectively. In our studies, however, amplicons were undetectable when this method was used. One possible explanation could be that, in their study, virus-inoculated food samples were processed according to a lengthy elution, filtration, and concentration procedure which, in itself, probably resulted in relatively "pure" viral suspensions with little or minimal co-

extracted inhibitors. RNA was then extracted from the viral suspensions with TRIzol® reagent and nucleic acids were purified with the QIAshredder™ homogenizer columns to further reduce the load of cellular debris and extraneous material. In our case, however, foods were solely processed with TRIzol® reagent and the isolated RNAs were directly added to the QIAshredder™ spin columns.

Studies conducted using magnetic Oligo (dT) beads demonstrated that they were the most efficient of all methods tested at purifying RNA suspensions following TRIzol® processing of spiked sliced deli-ham samples. In our studies, there was a 10-fold increase in test sensitivity when using these beads to purify RNA suspensions, as compared to the InstaGene™ matrix (Figures 8 & 10). These results are consistent with those reported by Kingsley *et al.* (100) who detected noroviruses and hepatitis A viruses from naturally-contaminated shellfish using Oligo (dT) beads to purify resuspended RNA pellets.

Therefore, the combination of TRIzol® reagent for viral concentration/RNA extraction and RNA purification with Oligo (dT) beads was used in subsequent studies to detect FCV from select food matrices. However, when applied to bread and lettuce samples, the method was relatively insensitive and results varied from those obtained with ham samples. Inhibition caused by plant-derived carbohydrates have been reported in the past (145), and others have shown a reduction in test sensitivity when applying viral concentration methods to lettuce as compared to other food commodities (99, 111). This may have been the case in our studies where sensitivity was the lowest when applying the method to lettuce samples. Therefore, our results indicated that the TRIzol®/Oligo d(T) bead methodology developed herein was not sensitive enough to be applied to the detection of NoV from food matrices such as lettuce, breads (for reasons

discussed previously), and foods similar in composition to breads (i.e., muffins, cakes). This is unfortunate seeing as such items are frequently implicated in outbreaks. For example, lettuce contaminated by irrigation water was involved in a 1988 hepatitis A outbreak that sickened 200 people (146). Despite obtaining reasonable sensitivity of detection when applying the methodology to ham samples, the goal of this work was to develop a method applicable to more than one food item. Therefore, the development of a different viral concentration and detection method was necessary.

As such, method development focused on the use of the Pathatrix™ system which is a novel technology that was developed to capture bacteria from select food matrices using immunomagnetic beads. The usefulness of these magnetic beads for virus concentration has been reported by several other groups because of their efficiency at separating intact virus particles from clinical, environmental, and food samples (114, 116, 147-150). Enhanced detection sensitivity reported by others may be due, in part, to the physical segregation of virus particles from extraneous molecules present in foods (PCR inhibitors). This eliminates the need for further virus purification steps so that isolated virus particles can be directly used in downstream detection applications (i.e., plaque assay, ELISA), or that the viral nucleic acids can be extracted and used in the RT-PCR. Gilpatrick *et al.* (116) were successful in detecting noroviruses from stool specimens with the use of paramagnetic beads conjugated to polyclonal antibodies derived from norovirus-like particle-immunized rabbits. However, this group reported that an assay with broader NoV specificity should be developed when monoclonal antibodies become available.

Due to the current lack of broadly reactive and specific monoclonal antibodies targeting the human noroviruses, we relied on using non-specific positively charged magnetic beads to capture viruses from foods. Our data indicates that these beads, used in the Pathatrix™ system, were able to concentrate both HAV and NoV from various food matrices based on the principle that a negatively charged viral capsid will interact with a positively charged bead surface. In developing this methodology, it was hoped that it would be applicable to capturing HAV and NoV without the need for virus-specific reagents (i.e., antibodies). Moreover, this method would be less time consuming and more consistent, since it wouldn't require the production of polyclonal antibodies and the standardization required when conjugating these antibodies to magnetic beads.

As expected, the Pathatrix™ system was successful in isolating hepatitis A virus from lettuce, strawberries, and green onions with detection limits at initial inocula of 1, 0.1, and 1 PFUs per 25 grams each, respectively (Figure 13). The captured virus was detectable after RNA extraction, RT-PCR amplification and gel electrophoresis. The sensitivity of this method would allow detection of HAV in foods at levels relevant to naturally-contaminated products, and consistent with the infectious dose of the virus (1). Moreover, results reported in our study are comparable to, or even better than, those obtained by others when using a viral concentration method combined with the RT-PCR for detection. For example, Guevremont *et al.* (102) reported detection limits of 1 $TCID_{50}$ HAV per 25g of fresh green onions when using a virus elution-concentration method while Shan *et al.* (124) used an immunomagnetic capture method and real-time PCR to detect as low as 0.5 PFU HAV in green onions and strawberries. Furthermore,

with our method, inhibitors associated with each food matrix were effectively separated from the virus, as indicated by the similar detection limits obtained with each food item.

Though successful at capturing HAV from foods, the Pathatrix™ methodology was less sensitive when applied to the isolation of noroviruses from select food matrices. Detection limits were at initial inocula of 10^2, 10^4, and 10^5 RT-PCR units per 25 g of lettuce, ham and strawberries, respectively (Figure 14). Perhaps these discrepancies can be attributed to differences in viral capsid charge which could influence the strength of interaction between the beads and the virus. The fact that HAV was efficiently recovered using the cationic particles may be a function of a particularly strong negative charge on the surface of this virus; indeed, HAV tends to adhere to surfaces quite tenaciously as compared to other enteric viruses (151).

The observed differences in detection limits between viruses could also be due to over- or under- estimations during NoV and/or HAV quantification. HAV concentration was calculated by both the plaque assay (PFUs per ml) and end point dilution (RT-PCR units per ml), and their titers were compared. Quantification by end point dilution usually yields more sensitive results than by the plaque assay, as has been mentioned by various investigators. For example, ratios of 23 PCR units per PFU (107) and 55.7 RT-PCR_{50} units per PFU (110), have been reported. These results are consistent with the fact that the RT-PCR is highly sensitive and can detect as little as a few copies of the NoV genome in a sample (141), regardless of whether the virus particle from which it was extracted was infectious or not. With the plaque assay, however, only infectious virus particles can be detected. For in-vitro-propagated HAV stocks, the particle-to-PFU ratios have been estimated to be as high as 1,000 virus particles per PFU (152). Surprisingly,

however, HAV titers calculated in these studies were the same by both methods of quantification so that the RT-PCR-unit-per-PFU ratio was 1. Perhaps the primers used to determine the end-point dilution during HAV quantification lacked sensitivity and provided an underestimation. As such, the primers we used for HAV quantification were tested against a primer set designed by Schwab *et al.*, (118) and the latter primers seemed to detect HAV in more outbreak food samples and spiked foods than the other ones (unpublished data). Therefore, it is possible that the primers designed by Schwab would have given more sensitive results than those used throughout our studies, so that an accurate titer could be calculated. In contrast, the NVp110/NVp36 primer set used to quantify noroviruses was previously reported as yielding the best detection limits when compared to other sets of published primers (111). Other factors affecting end-point dilutions could be attributed to discrepancies in thermocycling conditions between PCR machines and/or insufficiently optimized RT-PCR conditions (i.e., reaction mixture components).

To further investigate the possibility that method efficiency varied significantly between NoV and HAV due to miscalculations in viral titers, calculated viral concentrations were converted to a common unit of measure. As such, with each RT-PCR unit reported to represent 10-50 genomic copies (and assuming that there is 1 genomic copy per virus particle) (118, 153), test sensitivity for HAV and NoV in lettuce was compared by converting the detection limit of each virus into viral particles per 25 g. For example, the detection limit of HAV in lettuce was 1 PFU/25 g, which is equivalent to 10^3 viral particles, based on the estimation that there are 1000 virus particles per PFU (152). For NoV, the detection limit in lettuce was 10^2 RT-PCRu/25 g so that it is

assumed that there are approximately 10^3 genomic copies (=10^3 virus particles). In applying these conversions we would obtain 10^3 virus particles per 25 g of lettuce for both HAV and NoV. Although these calculations show that both viruses essentially have the same detection limit in lettuce, it still does not explain why HAV titers are not higher when expressed as RT-PCR units versus PFUs. Furthermore, these calculations do not seem applicable to our studies as seen when converting the detection limits obtained for virus-inoculated strawberries (in PFU or RT-PCRu) into genomic copies. Results from these conversions differ greatly at 10^2 and 10^6 virions of HAV and NoV per 25 g of strawberries, respectively.

Finally, when investigating the discrepancies in the results obtained for NoV and HAV, the source of each virus must also be considered; HAV stocks used were propagated in-vitro while NoV stocks came from clinical stool specimens. Perhaps there were molecules and debris from the stool suspensions that remained attached to the norovirus capsid, thereby affecting its access to the beads. In summary, it seems that the major differences in test sensitivity between the two viruses could be due to inaccuracies in viral quantification, clarification, differences in capsid charges (affecting the interaction with cationic beads), and/or inaccessibility to the viral capsid.

Another important difference between results obtained for HAV and NoV was that detection limits achieved in various HAV-inoculated foods were very similar, while test sensitivity fluctuated for each NoV-inoculated food matrix. For HAV, detection limits remained within a range of 0.1 to 1 PFUs per 25 g of strawberries, lettuce, and green onions, while NoV detection limits varied from 10^2, 10^4, and 10^6 RT-PCR units per 25 g of lettuce, ham, and strawberries, respectively (Figures 13 & 14, respectively). It is

possible that the variation in sensitivity between food items was due to differences in the elution of NoV from each food surface. When treating samples with TRIzol® reagent, the method was insensitive due to the inadequate purification of co-extracted molecules in the final RNA suspensions. However, the Pathatrix™ system effectively separated the virus from extraneous food particles (as shown with the consistent results obtained with HAV, irrelevant of the food matrix tested), so that test insensitivity is unlikely to be a problem of inhibition. Furthermore, detection limits were the poorest with FCV-inoculated lettuce samples (as compared to other foods tested) when using TRIzol® reagent but detection limits were the best in NoV-inoculated lettuce processed with the Pathatrix™ system. Therefore, it is highly unlikely that inhibitors co-isolated from foods are responsible for the insensitivity and inconsistency seen when using the Pathatrix™ technology to isolate NoV from inoculated foods.

With NoV and HAV belonging to different virus families, some of their physical and chemical properties will differ. These physico-chemical variations may have an impact on the interactions of each virus with food matrices when placed in buffers of different pH values. As such, the variability in the results obtained between the viruses and foods may be explained by differences in the isoelectric point (pI) of each virus and each food matrix. The isoelectric point (pI) is the pH at which a protein (i.e., virus particle or components of the food matrix) carries no net electrical charge. At a pH below the pI, proteins carry a net positive charge while a pH above the pI will give a protein a negative charge. The amino acid residues on the virus surface and the pH of the surrounding medium ultimately define the net surface charge on a virus. Several factors must be considered when using the Pathatrix™ system: 1) certain food constituents carry

a charge that varies with pH, 2) cationic beads used in the system remain positively charged at all times, and 3) the net charge of the viral capsid will fluctuate depending on the pH of the medium and the pI of the virus (109). The ideal situation in our methodology would be to adjust the pH of the elution buffer so that it is above the pI of both the virus (giving it a negative charge) and the food surface (giving it a negative charge). Thus, repulsion would occur between the virus and the food surface, while attraction would occur between the virus and the cationic beads.

The isoelectric point of the noroviruses ranges from 5.5 to 6.9, depending on the genogroup and strain (154). We observed that the test sensitivity was greater in spiked deli ham when using a saline solution at pH 7.2 than when using glycine buffer at a pH of 8.5. Conversely, using glycine buffer (pH 8.5) instead of the saline solution (pH 7.2) increased the test sensitivity when concentrating noroviruses from spiked strawberry and lettuce samples. However, the use of an elution buffer with a pH above the isoelectric point of the virus should have yielded much more sensitive detection limits as compared to the results obtained with HAV. It is not easily determined whether the buffer composition (EBSS vs. glycine) or the pH of the media (7.2 vs. 8.5), had a profound effect on these results.

Generally, for both viruses, test sensitivity was enhanced when using glycine buffer (pH 8.5) over EBSS media (pH 7.2). Perhaps EBSS has poor buffering capabilities and/or its neutral pH does not promote viral dissociation from food surfaces. It is likely that the pH of the glycine buffer (pH 8.5) used to elute HAV from foods was in the optimal range since the results obtained were very sensitive for all matrices. The

isoelectric point of HAV was not found in the literature, but it may be slightly higher than the one determined for the noroviruses.

In addition to the pH value of a buffer affecting virus-food surface interactions, the ionic strength of the media may also have had an effect on virus detachment from food surfaces. Although elution buffers made with varying concentrations of glycine and sodium chloride are the most widely used for viral elution from foods (99-102, 104), others exist as well. Schaldach *et al.* (155) recently reported that the ionic strength of media plays a critical role in influencing the adsorption and, consequently, the detachment of viruses from surfaces. For example, Penrod *et al.* (156) showed that the MS2 bacteriophage attaches more efficiently to surfaces in buffers with increasing ionic strength up until a maximum of 0.1 M is reached, and then attachment decreases above that point. In our studies, in an attempt to promote viral detachment from food surfaces, the elution buffer used contained a high concentration of glycine (0.5 M) as compared to certain other groups (102, 110, 111). The buffer composition used may have favored HAV detachment from foods while noroviruses were poorly eluted from the same surfaces. Perhaps the glycine concentration used (0.5 M) was above or below the maximum ionic strength needed to promote successful detachment of the virus from foods. Therefore, as seen in our studies, a host of parameters must be considered when developing methods to wash viruses from surfaces. Moreover, the interaction of cationic beads with the virus was an additional parameter to consider in these studies.

The cationic beads used in these studies were designed by Matrix MicroScience and, due to it being proprietary information, we did not know their concentration, size, or what gave them their positive charge. Thus we were unable to determine whether one or

more of these factors could have influenced viral recovery. Occasionally, with virus-inoculated lettuce and strawberries, amplicons which were usually detectable could not be seen while, in the same trial, amplicons from lower virus concentrations were detected. Although the reasons for these results are unclear, they may be explained by the fact that HAV is a particularly sticky virus that readily precipitates (99). Thus, in solution, the virus could clump together and, at higher concentrations, these clumps would bind to beads and obstruct their access to the magnet. At lower viral inputs, however, the clumps would be much smaller and the beads could retain ready access to the magnet. Although noroviruses have not been reported as being sticky viruses that aggregate in solution, the same theory could apply to them. However, the likelihood of this theory being correct would also depend on the ratio of bead to virus concentration. If the bead concentration was higher than that of the virus particles, there wouldn't be sufficient virus particles in the sample to cover the surface of all the beads. Without further information on the nature of the cationic beads used, the above theory remains speculative.

Though information on the cationic beads was lacking, the quantity needed to achieve the greatest sensitivity was assessed. An improvement in the detection limit was observed when 100 rather than 50 µl of bead suspension was added to HAV-inoculated lettuce samples. However, the sensitivity was not increased when adding 200 µl of beads to the samples. Perhaps the bead concentration in a 50 µl volume is not sufficiently elevated to allow uniform circulation of the beads throughout the entire sample. It's also possible that most of the beads were covered by food particles, leaving only a few to bind the virus. By doubling the bead concentration, beads were effectively dispersed

throughout the sample to a maximum saturation level without having any effect on virus capture. Similar trends in bead concentration were observed with HAV-inoculated strawberries, where the best detection limits were achieved with 200 µl (vs. 100) of beads. Perhaps an increase in bead concentration is needed with this food matrix, as berries release acids that could alter the pH of the elution buffer so that interactions between the cationic beads and the virus may be affected. The chances of an interaction between the virus and beads may increase by adding a higher concentration of the latter.

It was also thought that by homogenizing foods prior to processing, the cationic beads would have greater access to all of the food's surfaces, i.e., the interior and exterior food surfaces would become uniformly distributed in buffer. However, for each food matrix tested, the test sensitivity was compromised by the blending of foods prior to recirculation in the Pathatrix™ system. Following circulation, beads captured on the magnet of the Pathatrix™ machine were visibly reduced in all cases. By homogenizing the foods, the density of the buffer in which the food particles are suspended greatly increased. As such, bead recirculation throughout the food suspension became more difficult, resulting in reduced interactions between the beads and the virus, and a reduced accessibility to the magnet.

Once a method was standardized for the capture of viruses with the Pathatrix™ system, viral nucleic acids had to be extracted, amplified and detected. The method used to extract viral RNA played an important role in the consistency of detection. In initial studies, FCV end-point dilutions were always consistent when using a heat-release method, the QIAamp™ viral RNA mini-kit, or TRIzol® reagent for RNA extraction of serial 10-fold viral dilutions. However, when isolating viruses from foods with the

Pathatrix™ methodology, heating captured viruses to release RNA did not consistently allow viral detection. In contrast, using the QIAamp™ viral RNA mini-kit allowed consistent detection when viruses were isolated from foods. These results showed that when RNA was concentrated in the silica-gel-based membrane, the QIAamp™ kit effectively washed away contaminants co-extracted from foods.

Conclusions

The Pathatrix™ methodology described herein offers numerous advantages over other methods for the concentration of HAV from various food commodities. Not only are the reported detection limits highly sensitive, but the methodology allowed for viruses to be isolated from foods within 30 min, thus results were obtainable within one day. Obtaining rapid results during an outbreak is very important so that proper actions can be quickly implemented. Also, the method provided here is user-friendly and relatively hands-off for the technician, minimizing the chances of sample contamination from laboratory sources. Moreover, the Pathatrix™ system can accommodate up to five different food samples in a single run and allows for the testing of the entire 25 g sample, an amount that is commonly reported as being a standard analytical unit in Health Canada's Compendium of Analytical Methods (157). Furthermore, as shown in our competition studies, the cationic beads used in conjunction with the Pathatrix™ system are non-specific, and would allow for simultaneous multi-pathogen capture. This ability to co-capture various pathogens provides an advantage to researchers doing routine screening for multiple viruses and/or bacteria in foods, as these are often contaminated with more than one pathogen (158-160).

Although the NoV detection limits reported here were not nearly as sensitive as those obtained for HAV following Pathatrix™ isolation, the developed methodology was able to detect noroviruses in naturally-contaminated outbreak food samples. This suggests that the Pathatrix™ system is sufficiently sensitive to detect noroviruses at levels present in naturally-contaminated foods. Moreover, the reported norovirus titer may not be exact, resulting in inaccurate detection limits. Similarly, despite its relatively

poor detection limits in artificially-inoculated foods, the methodology described with the TRIzol® reagent was sufficiently sensitive in detecting noroviruses in outbreak foods.

When comparing both methods, however, it is clear that the Pathatrix™ system offers advantages over the TRIzol® methodology for the isolation of noroviruses from various food matrices. Furthermore, outbreak foods which could not be tested with TRIzol® (i.e., breads, potato salads) were effectively tested and the virus captured with the Pathatrix™ system. To-date, the only foods not able to be processed with the Pathatrix™ were those with thick consistencies such as dressings and mayonnaise. Therefore, this system allows for a broader range of foodstuff to be tested under the same processing conditions, which is a huge advantage in outbreak situations.

In conclusion, our studies have validated reports that the co-extraction of inhibitory molecules greatly affects the ability to detect viruses in select food matrices. Our findings have also contributed to advancements in the field of food virology by developing a novel method for the isolation of the most important foodborne viruses from select food matrices. Overall, the use of magnetic beads is perhaps one of the most promising techniques for both the isolation of pathogens from foods and for the removal of extraneous food particles from viral isolates. With foodborne disease being an on-going threat to public health, it is crucial that rapid, user-friendly, and universally applicable methods be developed and widely used by researchers. The Pathatrix™ methodology certainly fits these criteria by using non-specific positively-charged beads to capture various negatively-charged pathogens, making this protocol applicable to a wide variety of outbreak situations.

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Appendix A: Composition of buffers used for cell culture and virus propagation

	Reagents[a]	Maintenance Media[b]	Growth Media[b]
CrFK cells	10X MEM with Earle's salt	85 ml	85 ml
	Double distilled water	848 ml	768 ml
	Fetal bovine serum	20 ml	100 ml
	Penicillin/Streptomycin (100X)	10 ml	10 ml
	Sodium bicarbonate, 7.5%	27 ml	27 ml
	Non-essential amino acids, 100 mM	10 ml	10 ml
	pH	7.3	7.3
FRhK-4 cells	10X MEM with Earle's salt	100 ml	100 ml
	Double distilled water	825 ml	745 ml
	Fetal bovine serum	20 ml	100 ml
	MEM sodium pyruvate, 100 mM (100X) liquid	10 ml	10 ml
	L-glutamine, 200 mM	10 ml	10 ml
	Non-essential amino acids, 100 mM	10 ml	10 ml
	Penicillin/Streptomycin (100X)	10 ml	10 ml
	Sodium bicarbonate, 7.5%	15 ml	15 ml
	pH	7.3	7.3

[a] Purchasing information and catalogue numbers of each item are specified in the materials and methods section;

[b] Volume needed to make 1 L of buffer.

Appendix B: Composition of buffers used for plaque assays

a) Overlay media (Agarose-medium mixture)

Reagents[a]	Feline calicivirus	Hepatitis A virus
10X MEM with Eagle's salt	100 ml	90 ml
Double-distilled water	355 ml	183.3 ml
Fetal bovine serum	20 ml	18 ml
Non-essential amino acids, 100 mM	10 ml	9 ml
Sodium bicarbonate, 7.5%	12.5 ml	13.5 ml
Magnesium chloride	3.7 g	4.5 g
Gentamycin (50 µg/ml)	1 ml	0.9 ml
L-glutamine, 200 mM	XX[b]	9 ml
HEPES, 1.5 M	XX	9 ml
Kanamycin, 200 µg/ml in saline	XX	105.6 ml
Amphotericin B, 250 µg/ml	XX	4 ml
Type II Agarose (Sigma, cat.A-6877), 1.2%	6g/500 ml/DDW	6g/500 ml/DDW

[a] Purchasing information and catalogue numbers of each item are specified in the materials and methods section;

[b] Reagent not used for this virus.

b) Crystal Violet Solution

Crystal Violet - 2 gm
95% alcohol - 20 mL
Ammonium oxalate - 0.8 gm
Distilled water - 80 mL

Dissolve crystal violet in alcohol and the ammonium oxalate in water. Allow the ammonium oxalate solution to stand overnight or heat gently until dissolved. Mix the two solutions together and filter.

Appendix C: Composition of buffers used for gel electrophoresis

a) 0.5X TBE Buffer

10X TBE stock solution:

890 mM Boric Acid
20 mM EDTA, pH 8.0
890 mM Tris

To make 1 L (0.5X):

Add 50 ml of 10X TBE to 950 ml of DDW.

b) Ethidium bromide (5 µg/ml)

For gel staining:

Add 5 µl of ethidium bromide stock solution (10 mg/ml) to 100 ml of DDW or 0.5X TBE.

Teaching Assistant (BIO 3576) 2005
Faculty of Sciences, University of Ottawa, Ottawa, ON
Assisting Dr. Gabriel Blouin-Demers in the course of Animal Behaviour. Leading discussion groups on various topics in animal behaviour, evaluating student participation, marking midterms, proctoring the final exam.

Orthopaedic Assistant 2003, 2002
Foot & Sole Clinic, North Bay, ON
Biomechanical assessment of patients, assessing clients' needs and offering proper solutions, retails sales, transmission of product knowledge, measuring for compression hosiery.

French Language Monitor 2001-2003
Ottawa-Carleton School Board, Ottawa, ON
Teaching French to students (kindergarten to grade 12). Assisting with reading, spelling, composition and expression. Planning activities and lessons. Evaluating students.

Septic Re-Inspection Technician 2001
North Bay-Mattawa Conservation Authority, North Bay, ON
Septic system inspections at waterfront homes. Educating home owners on the need for preserving water quality. Organizing different fundraisers.

List of Publications

Papafragkou, E., **Plante, M.,** Mattison, K., Bidawid, S., Karthikeyan, K., Farber, J.M. and Jaykus, L.-A. (2008). Rapid and sensitive detection of hepatitis A virus in representative food matrices. *J. Virol Methods*. 147: 177-187.

List of Major Presentations

Platform presentations

Plante, M., Farber, J.M., Bidawid, S. and K. Mattison. Development of methods for the detection of enteric viruses in foods. Health Canada Science Forum, Ottawa, ON (Octobter 2005)

Plante, M., Bidawid, S., and K. Karthikeyan. Development of methods for the detection of noroviruses in foods. As part of Health Canada's seminar series to scientists and research technicians, Health Canada, Food Directorate, Bureau of Microbial Hazards, Ottawa, ON (April 2005)

Plante, M., Farber, J.M. and S. Bidawid. Development of methods for norovirus concentration and rapid detection in foods. Presentation given to fellow graduate students. researchers and staff of the department of Biochemistry, Microbiology and Immunology as part of the requirements of the Masters program. University of Ottawa, Faculty of Medicine, Ottawa, ON (January 2004)

Poster Presentations

Mattison, K., **Plante, M.,** Bidawid, S. and J.M. Farber. Rapid extraction and detection of viruses from food samples using the Pathatrix system. Federal Food Safety and Nutrition Research Meeting, Ottawa, ON (October 2006)

Plante, M., Karthikeyan, K., Bidawid, S., Mattison, K. and J.M. Farber. Rapid extraction and detection of hepatitis A virus from select food samples. Health Canada Science Forum 2005, Ottawa, ON (October 2005); International Association of Food Protection (IAFP) Conference 2005, Baltimore, MD, USA (August 2005)

Plante, M., Karthikeyan, K., Bidawid, S., Mattison, K. and J.M. Farber. Development of methods for norovirus detection from various outbreak foods. Health Canada Science Forum 2005, Ottawa, ON

(October 2005); International Association of Food Protection (IAFP) Conference 2005, Baltimore, MD, USA (August 2005)

Plante, M., Malik, N., Karthikeyan, K., Bidawid, S. and J.M. Farber. Development of methods for norovirus concentration and rapid detection in foods. Graduate student poster day 2004, University of Ottawa, Faculty of Medicine, Department of Biochemistry, Microbiology and Immunology, Ottawa, ON (April 2004)

Extracurricular Activities

Workshop creator and animator for "Mini-cours de médicine" which promote the study of medicine to francophone high school students, Montfort Hospital (Ottawa, ON) and Centre des compagnons (North Bay, ON)	2007
Various city intramural sports leagues	1999 - present
High School graduation committee	1999
High School class president	1999